



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/29, 15/82, A01N 65/00, A01H 5/00</b>		A1	(11) International Publication Number: <b>WO 96/35790</b> (43) International Publication Date: 14 November 1996 (14.11.96)
(21) International Application Number: <b>PCT/GB96/01155</b>		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 13 May 1996 (13.05.96)			
(30) Priority Data: 9509575.8 11 May 1995 (11.05.95) GB			
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(54) Title: PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

## (57) Abstract

The tomato *Cf-4* gene has been isolated by positional cloning and its sequence provided, along with the encoded amino acid sequence. DNA encoding the polypeptide, alleles, mutants and derivatives thereof, and DNA encoding amino acid sequences showing a significant degree of homology thereto may be introduced into plant cells and the encoded polypeptide expressed, conferring pathogen resistance on plants comprising such cells and descendants thereof. The *Cf-4* sequence shows a high degree of homology to *Cf-9* and comprises leucine rich repeats.

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## PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

The present invention relates to pathogen resistance in plants and more particularly the identification and use of pathogen resistance genes.

5 It is based on cloning of the tomato *Cf-4* gene.

Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, since they are usually grown as genetically uniform monocultures. However, plants 10 have evolved an array of both preexisting and inducible defences which pathogens must circumvent, especially those pathogens that derive their nutrition from an intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to 15 be compatible, but if the plant is resistant, the interaction is said to be incompatible.

Race specific resistance is often (though not exclusively) specified by dominant R genes. When pathogens mutate to overcome R genes, the mutations are 20 often recessive. For R genes to function, there must also be a corresponding gene in the pathogen termed an avirulence gene (Avr). To become virulent, pathogens (fungi, bacteria or viruses) must no longer produce a product that triggers R gene-dependent defence 25 mechanisms (Flor, 1971). One model, often termed the elicitor/receptor model, is that R genes encode products which enable plants to detect the presence of pathogens, provided the pathogen carries the

corresponding Avr gene (Gabriel and Rolfe, 1990). This recognition is subsequently transduced into the activation of a defence response.

The characterization of two fungal avirulence genes from the tomato leaf mould pathogen *Cladosporium fulvum* has been reported. The *Avr9* and *Avr4* genes encode small cysteine rich peptides (van Kan et al., 1991; Joosten et al., 1995) which, in their mature processed forms, are composed of 28 and 106 amino acid residues respectively. *Avr9* and *Avr4* confer avirulence to races of *C. fulvum* on tomato lines carrying the *R* genes *Cf-9* and *Cf-4* respectively. We have shown that these two *R* genes are genetically tightly linked, or possibly allelic (Jones et al., 1993; Balint-Kurti et al., 1994). We isolated the *Cf-9* gene by transposon tagging (Jones et al., 1994; PCT/GB94/02812, published as WO 95/18230) and we report here the positional cloning of *Cf-4* using more refined genetic analysis and *Cf-9* DNA as a probe. The availability of cloned avirulence genes and their cognate resistance genes may ultimately be exploited to engineer broad-based and durable disease resistance in a wide range of crop plants (de Wit, 1992; Staskawicz et al., 1995).

In plants gene isolation has been achieved by two main approaches, positional cloning and transposon tagging. Several plant genes have been successfully isolated by positional cloning (reviewed in Tanksley et al., 1995) including several *R* genes i.e. *Pto* (Martin

et al., 1993) and *Cf-2* from tomato (Dixon et al., 1996) and *RPS2* and *RPM1* from *Arabidopsis* (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995). Most positional cloning strategies have relied heavily on 5 the use of restriction fragment length polymorphism (RFLP) markers. Recently however, PCR-based strategies have been developed which are capable of detecting more subtle DNA sequence variation allowing a much greater number of DNA sequences to be inspected for 10 polymorphism. These techniques include random amplified polymorphic DNA (RAPDs, Williams et al., 1990) and amplified restriction fragment polymorphism analysis (AFLP, Zabeau and Vos, 1992, EP-A-92402629.7; Vos et al. 1995; Thomas et al., 1995) and should 15 expedite plant gene isolation by positional cloning strategies. Transposon tagging has been used to isolate the *N* gene from tobacco (Whitham et al., 1994), *L*<sup>6</sup> from flax (Lawrence et al., 1995) and *Cf-9* from tomato (Jones et al., 1994; PCT/GB94/02812, published 20 as WO 95/18230).

Amino acid sequence comparisons of plant *R* genes has shown they currently constitute two major classes. All except PTO appear to contain leucine rich repeat (LRR) motifs but the *R* genes *L*<sup>6</sup>, *N*, *RPM1* and *RPS2* 25 appear to have additional domains not present in *Cf-9* (Staskawicz et al., 1995) and *Cf-2* (Dixon et al., 1996). Furthermore, *L*<sup>6</sup>, *N* and *RPS2* are probably located intracellularly in contrast to *Cf-9* and *Cf-2*

which are composed largely of LRRs and are predicted to be predominantly extra-cytoplasmic membrane-anchored proteins. Our analysis shows that the predicted Cf-4 protein is highly homologous to Cf-9.

WO93/11241 reports the sequence of a gene encoding a polygalacturonase inhibitor protein (PGIP) that has some homology with Cf-9 and, as we have now discovered, Cf-4 (the subject of the present invention). Cf-9, Cf-4 and others (Cf-5, -2 etc.) are termed by those skilled in the art "pathogen resistance genes" or "disease resistance genes". PGIP-encoding genes are not pathogen resistance genes. A pathogen resistance gene (R) enables a plant to detect the presence of a pathogen expressing a corresponding avirulence gene (Avr). When the pathogen is detected, a defence response such as the hypersensitive response (HR) is activated. By such means a plant may deprive the pathogen of living cells by localised cell death at sites of attempted pathogen ingress. On the other hand, the PGIP gene of WO93/11241 (for example) is a gene of the kind that is induced in the plant defence response resulting from detection of a pathogen by an R gene.

Thus, a pathogen resistance gene may be envisaged as encoding a receptor to a pathogen-derived and Avr dependent molecule. In this way it may be likened to the RADAR of a plant for detection of a pathogen, whereas PGIP is involved in the defence the plant.

mounts to the pathogen once detected and is not a pathogen resistance gene. Expression of a pathogen resistance gene in a plant causes activation of a defence response in the plant. This may be upon 5 contact of the plant with a pathogen or a corresponding elicitor molecule, though the possibility of causing activation by over-expression of the resistance gene in the absence of elicitor has been reported. The defence response may be activated locally, e.g. at a site of 10 contact of the plant with pathogen or elicitor molecule, or systemically. Activation of a defence response in a plant expressing a pathogen resistance gene may be caused upon contact of the plant with an appropriate, corresponding elicitor molecule, e.g. as 15 produced by a *Cladosporium fulvum* *avr* gene as discussed. The elicitor may be contained in an extract of a pathogen such as *Cladosporium fulvum*, or may be wholly or partially purified and may be wholly or partially synthetic. An elicitor molecule may be said 20 to "correspond" if it is a suitable ligand for the R gene product to elicit activation of a defence response.

The "Cf-x"/"Avr<sub>x</sub>" terminology is standard in the art. The Cf resistance genes and corresponding fungal 25 avirulence genes (Avr) were originally defined genetically as interacting pairs of genes whose measurable activities fall into mutually exclusive interacting pairs. Avr9 elicits a necrotic response on

*Cf-9* containing tomatoes but no response on *Cf-4* containing tomatoes, the moiety recognised by *Cf-4* being different from that recognised by *Cf-9*.

5 Expression of *Cf-4* function in a plant may be determined by investigating compatibility of various *C. fulvum* races.

A race of *C. fulvum* that carries functional copies of all known *Avr* genes (race 0) will grow (compatible) only on a tomato which lacks all the *Cf* genes. It will not grow (incompatible) on a plant carrying any functional *Cf* gene. If the *C. fulvum* race lacks a functional *Avr4* gene (race 4) it will be able to grow not only on a plant lacking any *Cf* genes but also a plant carrying the *Cf-4* gene. A race also 15 lacking a functional *Avr2* gene (race 2,4) will also be able to grow on a plant carrying the *Cf-2* gene. A race only lacking a functional *Avr2* gene (race 2) will not be able to grow on a plant carrying *Cf-4*. Similarly, a *C. fulvum* race 5 (lacking a functional *Avr5* gene) will 20 not be able to grow on a plant carrying a *Cf-4* gene. Neither a race 4 nor a race 2,4 will be able to grow on a plant carrying any of the other *Cf* genes. Various races are commonly available in the art, e.g. from the Research Institute for Plant Protection (IPO-DLO), PO 25 Box 9060, 6700 GW Wageningen, The Netherlands. A race 4 is available under accession number IPO10379 and a race 2,4 available under Accession number IPO50379.

The *Cf-2* gene is the subject of PCT/GB96/00785

filed 1 April 1996 by John Innes Centre Innovations Limited and claiming priority from GB9506658.5.

We have now isolated a tomato gene, *Cf-4*, which confer resistance against the fungus *Cladosporium fulvum* and we have sequenced the DNA and deduced the amino acid sequence. The DNA sequence of the tomato *Cf-4* gene is shown in Figure 5 (SEQ ID NO. 1) and the deduced amino acid sequence shown in Figure 6 (SEQ ID NO 2).

According to one aspect, the present invention provides a nucleic acid isolate encoding a pathogen resistance gene, the gene being characterized in that it encodes the amino acid sequence shown in SEQ ID NO. 2, or a fragment thereof, or an amino acid sequence showing a significant degree of homology thereto. This may be a greater degree of sequence identity with the amino acid sequence of SEQ ID NO. 2 than is shown by the amino acid sequence of any of *Cf-9*, *Cf-2* and *Cf-5*. This may be at least about 95% identity.

Most preferably the nucleic acid encodes the amino acid sequence shown in SEQ ID NO. 2 in which case the nucleic acid isolate may comprise nucleic acid having the sequence shown in SEQ ID NO 1 or sufficient part to encode the desired polypeptide (eg from the initiating methionine codon to the first in frame downstream stop codon). In one embodiment DNA comprises a sequence of nucleotides which are the nucleotides 201 to 2618 of SEQ ID NO. 1, or a mutant,

derivative or allele thereof.

A further aspect of the invention provides a nucleic acid isolate encoding a pathogen resistance gene, or a fragment thereof, obtainable by screening a 5 DNA library with a probe comprising nucleotides 201 to 2618 of SEQ ID No. 1, or a fragment, derivative, mutant or allele thereof, and isolating DNA which encodes a polypeptide able to confer pathogen resistance to a plant, such as resistance to races of *Cladosporium* 10 *fulvum* (eg. expressing Avr4). The plant may be tomato. Suitable techniques are well known in the art.

Thus, the present invention also provides a method of identifying and/or isolating nucleic acid encoding a pathogen resistance gene comprising probing 15 *candidaet* (or "target") nucleic acid with nucleic acid which has a sequence of nucleotides which encodes an amino acid sequence shown in SEQ ID NO. 2, which is complementary to an encoding sequence or which encodes a fragment of either an encoding sequence or a sequence 20 complementary to an encoding sequence. The candidate nucleic acid (which may be, for instance, cDNA or genomic DNA) may be derived from any cell or organism which may contain or is suspected of containing nucleic acid encoding a pathogen resistance gene. A preferred 25 nucleotide sequence appears in SEQ ID NO. 1. Sequences complementary to the sequence shown, and fragments thereof, may be used.

Preferred conditions for probing are those which

are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain.

5 Nucleic acid according to the present invention may encode the amino acid sequence shown in SEQ ID No. 2 or a mutant, derivative or allele of the sequence provided. Preferred mutants, derivatives and alleles are those which retain a functional characteristic of the protein encoded by the wild-type gene, especially the ability to confer pathogen resistance, and most especially the ability to confer resistance against a 10 pathogen expressing the Avr4 elicitor molecule.

15 Changes to a sequence, to produce a mutant or derivative, may be by one or more of insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the insertion, deletion or substitution of one or more amino acids. Of course, 20 changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

25 Also provided by an aspect of the present invention is nucleic acid comprising a sequence of nucleotides complementary to a nucleotide sequence hybridisable with any encoding sequence provided herein. Another way of looking at this would be for nucleic acid according to this aspect to be

hybridisable with a nucleotide sequence complementary to any encoding sequence provided herein. Of course, DNA is generally double-stranded and blotting techniques such as Southern hybridisation are often 5 performed following separation of the strands without a distinction being drawn between which of the strands is hybridising. Preferably the hybridisable nucleic acid or its complement encode a polypeptide able to confer pathogen resistance on a host, i.e. includes a pathogen 10 resistance gene. Preferred conditions for hybridisation are familiar to those skilled in the art, but are generally stringent enough for there to be positive hybridisation between the sequences of interest to the exclusion of other sequences.

15 Nucleic acid according to the present invention, for instance mutants, derivatives and alleles of the specific sequences disclosed herein, may be distinguished from Cf-9, Cf-2 and/or Cf-5 by one or more of the following, or other features directly 20 derivable from comparison of the Cf-4 amino acid sequence with the respective other sequence or by observation of gene function:

- the amino acid sequence having at least about 95% identity with that of SEQ ID NO. 2;
- 25 - eliciting a defence response, in a plant expressing the nucleic acid, upon contact of the plant with Avr4 elicitor molecule, e.g. as provided by a *Cladosporium fulvum* race expressing Avr4 (available in

the art);

- not eliciting a defence response, in a plant expressing the nucleic acid, upon contact of the plant with the *C. fulvum* race 4 deposited at and available

5 from the Research Institute for Plant Protection (IPO-DLO), PO Box 9060, 6700 GW Wageningen, The Netherlands, under accession number IPO10379, or an extract thereof;

- not eliciting a defence response in a plant expressing the nucleic acid, upon contact of the plant

10 with the *C. fulvum* race 2,4 deposited at and available from the same institute under Accession number IPO50379, or an extract thereof;

- eliciting a defence response, in a plant expressing the nucleic acid, upon contact of the plant

15 with Avr4 elicitor molecule, e.g. as provided by a *Cladosporium fulvum* race or other organism expressing Avr4, amino acid and encoding nucleic acid sequences of which are given in WO95/31564 SEQ ID NO. 13, and Figure 4 herein;

20 - not eliciting a defence response, in a plant expressing the nucleic acid, upon contact of the plant with Avr9 elicitor molecule, e.g. as provided by a *Cladosporium fulvum* race or other organism expressing Avr9 (de Wit, 1992), the amino acid and encoding

25 nucleic acid sequences of chimaeric forms of which are given for example in WO95/18230 as SEQ ID NO 3 and in WO95/31564 as SEQ ID NO 4;

- not eliciting a defence response, in a plant

expressing the nucleic acid, upon contact of the plant with Avr2 elicitor molecule, e.g. as provided by a *Cladosporium fulvum* race or other organism expressing Avr2;

5 - not eliciting a defence response, in a plant expressing the nucleic acid, upon contact of the plant with Avr5 elicitor molecule, e.g. as provided by a *Cladosporium fulvum* race or other organism expressing Avr5;

10 - eliciting a defence response, in a plant expressing the nucleic acid and a corresponding elicitor molecule, at an earlier developmental stage than the defence response elicited in such a plant expressing the Cf-9 gene and corresponding Avr9

15 molecule;

- eliciting a defence response in a non-photosynthetic tissue (e.g. root) of a plant expressing the nucleic acid, upon contact of the plant with corresponding elicitor molecule, such as Avr4;

20 - comprising the number of leucine rich repeats (LRR's) identifiable from the sequence information provided herein for Cf-4.

The Cf-4 polypeptide, and others such as Cf-9, Cf-2 and Cf-5, may be distinguished from the products 25 of other pathogen resistance genes by being putative transmembrane proteins. The gene product of the *Arabidopsis RPP5* gene, for example, is a putative cytoplasmic protein.

A nucleic acid isolate according to the invention may encode a pathogen resistance gene whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides 5 encoding a polypeptide comprising the sequence of amino acids shown in SEQ ID NO. 2.

The activation may be upon contact of the plant with a pathogen or corresponding elicitor molecule.

The sequence of nucleotides may comprise an 10 encoding sequence shown in SEQ ID NO. 1. For example, the sequence may comprise nucleotides 201-2619 of SEQ ID NO. 1.

The nucleic acid may include a sequence of nucleotides comprising an allele, derivative or mutant, 15 by way of addition, insertion, deletion or substitution of one or more nucleotides, of an encoding sequence shown in SEQ ID NO. 1.

Nucleic acid according to the present invention may encode a pathogen resistance gene whose expression 20 in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide, the polypeptide comprising an amino acid sequence which comprises an allele, derivative or mutant, by way of addition, insertion, 25 deletion or substitution of one or more amino acids, of the amino acid sequence shown in SEQ ID NO. 2.

The nucleic acid isolate, which may contain the DNA encoding the amino acid sequence of SEQ ID No. 2 or

an amino acid sequence showing a significant degree of homology thereto as genomic DNA or cDNA, may be in the form of a recombinant vector, for example a phage or cosmid vector. The DNA may be under the control of an 5 appropriate promoter and regulatory elements for expression in a host cell, for example a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of genomic DNA this may be under the control of an 10 appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or 15 constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, 20 *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, 25 sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

The disclosures of Sambrook et al. and Ausubel et al., along with all other documents cited in the present text are incorporated herein by reference.

Nucleic acid molecules and vectors according to 5 the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of nucleic acid or genes of the species of interest or origin other than the sequence 10 encoding a polypeptide with the required function.

Nucleic acid according to the present invention may comprise cDNA, RNA, genomic DNA and may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

15 When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted may be assembled within a construct which contains effective regulatory elements 20 to promote transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material may or may not occur according to different embodiments 25 of the invention. Finally, as far as plants are concerned the target cell type should be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment.

containing pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a 5 disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, Bevan, 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, E-A-434616) microinjection (WO 92/09696, WO 94/00583, 10 EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611). *Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. Although 15 *Agrobacterium* has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a 20 combination of different techniques may be employed to enhance the efficiency of the transformation process, eg. bombardment with *Agrobacterium* coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co- 25 cultivation with *Agrobacterium* (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the

experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to 5 introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

A *Cf-4* gene and modified versions thereof, e.g. encoding a protein showing a significant degree of homology to the protein product of the *Cf-4* gene, 10 alleles, mutants and derivatives thereof, may be used to confer resistance in plants, in particular tomatoes, to a pathogen such as *C. fulvum*. This may include cloned DNA from *Lycopersicon hirsutum* which has the same chromosomal location as the *Cf-4* gene or any 15 subcloned fragment thereof. For this purpose a vector as described above may be used for the production of a transgenic plant. Such a plant may possess pathogen resistance conferred by the *Cf-4* gene.

The invention thus further encompasses a host 20 cell transformed with such a vector, especially a plant or a microbial cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome.

25 A vector comprising nucleic acid according to the present invention need not include a promoter, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the

genome.

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a promoter for control of expression of the encoded polypeptide. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell. Such introduction may be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The polypeptide encoded by the introduced nucleic acid may then be expressed.

A plant which comprises a plant cell according to the invention is also provided, along with any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

The invention further provides a method of comprising expression from nucleic acid encoding the amino acid sequence SEQ. ID No. 2, or a mutant, allele or derivative thereof, or a significantly homologous amino acid sequence, within cells of a plant (thereby producing the encoded polypeptide), following an

earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Such a method may confer pathogen resistance on the plant. This may be in combination with the *Avr4* gene according 5 to any of the methods described in WO91/15585 (Mogen) or, more preferably, PCT/GB95/01075, published as WO95/31564, or any other gene involved in conferring pathogen resistance.

The *Cf-4*, *Cf-9* and *Cf-2* genes function in a 10 similar manner in that they confer resistance to tomato that prevents growth of the tomato leaf mould *C. fulvum*. They do, however, work by the recognition of different *Avr* products and have subtle differences in the speed with which they stop growth of the pathogen 15 and stimulate a resistance response (Hammond-Kosack and Jones 1994, Ashfield et al. 1994). These differences may be exploited to optimise applications disclosed herein and in WO 95/31564 (above).

A gene stably incorporated into the genome of a 20 plant is passed from generation to generation to descendants of the plant, cells of which descendants may express the encoded polypeptide and so may have enhanced pathogen resistance. Pathogen resistance may be determined by assessing compatibility of a pathogen 25 (eg. *Cladosporium fulvum*) or using recombinant expression of a pathogen avirulence gene, such as *Avr-4* or delivery of the *Avr-4* gene product, for example in the form of a recombinant virus as described herein.

Sequencing of the *Cf-4* gene has shown that like the *Cf-9* gene (PCT/GB94/02812; Jones et al., 1994) and *Cf-2* (PCT/GB96/00785; Dixon et al., 1996) it includes DNA sequence encoding leucine-rich repeat (LRR) regions 5 and homology searching has revealed strong homologies to other genes containing LRRs. All three genes exhibit similar general features and as such represent a new class of disease resistance genes separate from other disease resistance genes characterised to date.

According to a further aspect, the present invention provides a method of identifying a plant pathogen resistance gene comprising use of an oligonucleotide which comprises a sequence or sequences that are conserved between pathogen resistance genes, 15 such as *Cf-9* and *Cf-4*, and/or *Cf-4* and *Cf-2*, to search for new resistance genes. Thus, a method of obtaining nucleic acid comprising a pathogen resistance gene (encoding a polypeptide able to confer pathogen resistance) is provided, comprising hybridisation of an 20 oligonucleotide (details of which are discussed herein) or a nucleic acid molecule comprising such an oligonucleotide to target/candidate nucleic acid. Target or candidate nucleic acid may, for example, comprise a genomic or cDNA library obtainable from an 25 organism known to encode a pathogen resistance gene. Clones that hybridise may be identified and target/candidate nucleic acid isolated for further investigation and/or use.

Hybridisation may involve probing nucleic acid and identifying positive hybridisation under suitably stringent conditions (in accordance with known techniques) and/or use of oligonucleotides as primers 5 in a method of nucleic acid amplification, such as PCR. For probing, preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further. It is well known in 10 the art to increase stringency of hybridisation gradually until only a few positive clones remain.

As an alternative to probing, though still employing nucleic acid hybridisation, oligonucleotides designed to amplify DNA sequences may be used in PCR 15 reactions or other methods involving amplification of nucleic acid, using routine procedures. See for instance "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, 1990, Academic Press, New York.

20 Preferred amino acid sequences suitable for use in the design of probes or PCR primers are sequences conserved (completely, substantially or partly) between polypeptides able to confer pathogen resistance, such as those encoded by *Cf-4* and *Cf-9*, and/or *Cf-4* and *Cf-2* 25 and/or *Cf-4*, *Cf-9* and *Cf-2*.

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code,

and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived.

Preferably an oligonucleotide in accordance with the invention, e.g. for use in nucleic acid amplification, has about 10 or fewer codons (e.g. 6, 7 or 8), i.e. is about 30 or fewer nucleotides in length (e.g. 18, 21 or 24).

Assessment of whether or not a PCR product corresponds to a resistance gene may be conducted in various ways. A PCR band may contain a complex mix of products. Individual products may be cloned and each screened for linkage to known disease resistance genes that are segregating in progeny that showed a polymorphism for this probe. Alternatively, the PCR product may be treated in a way that enables one to display the polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the resistance gene being preselected prior to cloning. Once a candidate PCR band has been cloned and shown to be linked to a known resistance gene, it may be used to isolate clones which may be inspected for other features and homologies to Cf-9, Cf-2, Cf-4 or other related gene. It may subsequently be analysed by transformation to assess its function on introduction into a disease sensitive variety of the plant of interest. Alternatively, the PCR band or sequences derived by analysing it may be used to assist plant breeders in monitoring the segregation of a useful

resistance gene.

These techniques are of general applicability to the identification of pathogen resistance genes in plants. Examples of the type of genes that can be 5 identified in this way include *Phytophthora* resistance in potatoes, mildew resistance and rust resistance in cereals such as barley and maize, rust resistance in *Antirrhinum* and flax, downy mildew resistance in lettuce and *Arabidopsis*, virus resistance in potato, 10 tomato and tobacco, nematode resistance in tomato, resistance to bacterial pathogens in *Arabidopsis* and tomato and *Xanthomonas* resistance in peppers.

Once a pathogen resistance gene has been identified, it may be reintroduced into plant cells 15 using techniques well known to those skilled in the art to produce transgenic plants. According to a further aspect, the present invention provides a DNA isolate encoding the protein product of a plant pathogen resistance gene which has been identified by use of the 20 presence therein of LRRs or, in particular, by the technique defined above.

According to yet a further aspect, the invention provides transgenic plants, in particular crop plants, which have been engineered to carry pathogen resistance 25 genes which have been identified by the presence of LRRs or by nucleic acid hybridisation as disclosed.

Examples of plants according to the present invention include tobacco, cucurbits, carrot, vegetable

brassica, lettuce, strawberry, oilseed brassica, sugar beet, wheat, barley, maize, rice, soyabeans, peas, sorghum, sunflower, tomato, potato, pepper, chrysanthemum, carnation, poplar, eucalyptus and pine.

5 Modifications to and further aspects and embodiments of the invention will be apparent to those skilled in the art. All documents mentioned herein are incorporated by reference. The term "comprises" should be interpreted herein as meaning "includes" or "has",  
10 not "consists of".

As already indicated, the present invention is based on the cloning and sequencing of the tomato Cf-4 genes and this experimental work is described in more detail below with reference to the following figures.

15 Figure 1 shows a Southern blot of *Bgl*III digested DNA isolated from near isogenic lines (NILs) of *L. esculentum* cv. Moneymaker containing either the Cf-9 (Cf9) or Cf-4 (Cf4) resistance genes, a line containing no known resistance genes to *C. fulvum* (Cf0), and  
20 another line (Cf1, *L. esculentum* cv. Stirling Castle) containing the Cf-1 resistance gene which has also been mapped to the short arm of tomato chromosome 1. The location of the 6.7 kbp *Bgl*III band corresponding to the Cf-9 gene and 3 *Bgl*III bands specific to Cf-4 containing  
25 lines and described in the text, are also shown.

Figure 2 shows in schematic form the three classes of recombinants identified in our analysis of 5

disease-sensitive recombinants (V408, V512, V514, V516 and V517) from the *Cf-4/Cf-9* transheterozygote testcross population. The *Cf-4* locus is depicted on the upper line and *Cf-9* on the lower line. The 5 location of two AFLP markers which flank *Cf-9* (M1 and M2, Thomas et al., 1995) are shown; one of these markers (M2) is also present at the *Cf-4* locus. Two other members of the multigene family located distal to *Cf-9* (shown here as genes A and B) which are present in 10 some disease-sensitive recombinants are also shown. Recombination breakpoints in each of the 3 classes which have been deduced by Southern hybridization and AFLP analysis are represented by broken lines.

15 Figure 3 shows a physical map of the *Cf-4* locus deduced from 3 overlapping cosmids (4/5-108, 4/5-129 and 4/5-138) isolated from the *Cf-4/Cf-5* cosmid library. The physical extent of each cosmid is shown schematically and the location of 3 *Cf-9* homologous sequences determined by hybridization and sequence 20 analysis are represented by black boxes. The transcriptional polarity of each gene is indicated by an arrow. The location of the *Cf-4* gene is also shown. Restriction enzyme sites for the following enzymes are also indicated; *Bgl*II, *Pst*I, *Eco*RI and *Xba*I. The 25 location of the 3 *Bgl*II fragments (6.4 kbp, 3.2 kbp and 1.8 kbp) showing high homology to a probe derived from the 5' end of *Cf-9* (see Figure 1) are indicated by arrows. M2 represents an AFLP marker originally

identified at the *Cf-9* locus also present at the *Cf-4* locus.

Figure 4 shows the nucleic acid (in double-stranded form) and deduced amino acid sequence of a *ClaI/SalI* DNA fragment encoding the *PR1a* signal peptide sequence fused to a sequence proposed to encode the mature processed form of *C. fulvum* AVR4. Translation initiation codon at nucleotide 5, termination codon beginning at nucleotide 413. Amino acids 1-30 represent the signal peptide sequence and amino acids 31-136 the mature AVR4 peptide.

Figure 5 shows a genomic DNA sequence of *Cf-4* (SEQ ID No. 1). Features of the sequence include; translation start site at nucleotide 201; translation stop beginning at nucleotide 2619; consensus polyadenylation sequence (AATAAA) beginning at nucleotide 2835; splice donor sequence in 3' untranslated sequence at nucleotide 2641; splice acceptor sequence ending at nucleotide 2755; proposed site of polyadenylation at nucleotide 2955.

Figure 6 shows the predicted amino acid sequence of the *Cf-4* gene (SEQ ID NO 2) according to the single letter amino acid code. The predicted protein sequence is composed of a primary translation product of 806 amino acids; signal peptide sequence amino acids 1-23; mature peptide amino acids 24-806.

Figure 7 shows the seven proposed structural domains A-G of the *Cf-4* protein as described for *Cf-9*

by Jones et al. (1994) and discussed in the text. Deletions in Cf-4 relative to Cf-9 are indicated by a dot. The majority of amino acids which distinguish the two proteins are located in their respective N-termini and are indicated in the figure as bold characters. Potential N-glycosylation sequences are shown underlined.

Figure 8 shows the two regions of major sequence divergence between the Cf-4 and Cf-9 sequences. (A) 10 Alignment of Cf-9 amino acids 40-79 of domain B(upper line) with amino acids 40-69 of Cf-4 (lower line). (B) Alignment of Cf-9 amino acids 285-426 (LRRs 9-14) with amino acids 274-369 of Cf-4 (LRRs 9-12).

Figure 9 shows the structure of four binary 15 vector plasmids used extensively for the expression of transgenes reported here. LB and RB correspond to the left and right borders of the T-DNA in each vector. Plant transformation marker genes were located at the LB end and were either the neomycin phosphotransferase 20 gene (NPT) or a gene from *Streptomyces hygroscopicus* conferring resistance to phosphinotricin (BAR). Transformation marker genes were either under control of the cauliflower mosaic virus 35S promoter (35S) or the nopaline synthase gene promoter from *A. 25 tumefaciens* (pnos). Transcription termination signals were provided by the *A. tumefaciens* octopine synthase gene 3' sequence (ocs3'). SLJ7291, SLJ7292 and SLJ755A all contain polylinker sequences with sites for the

restriction enzymes indicated in the figure. The polyliner sequence was derived from a modified pBluescript plasmid as described by Jones et al. (1992). The transcriptional orientation of all genes 5 is indicated by an arrow. All manipulations were performed as described by Jones et al. (1992). SLJ10080 was derived from the binary cosmid vector pCLD04541 by removing the *Cla*I site in the polylinker sequence by linearization with *Cla*I, T4 DNA polymerase 10 treatment and self ligation. The 35S:*uidA* cassette from SLJ4K1 was cloned into the resulting plasmid as an *Eco*RI/*Bam*HI fragment. *Cf*-4 and *Cf*-9 coding sequences for transient expression studies were cloned in as *Cla*I/*Bam*HI fragments replacing the *uidA* gene (see 15 Figure 10). Plasmids SLJ755A and pCLD04541, and their derivatives, contain a lambda phage sequence (cos) for use as cosmid cloning vectors.

Figure 10 binary vectors for expression of *Cf*-9 (pCf9/10080) and *Cf*-4 (pCf4/10080) in transient assays.

20 See text for details of each construct.

Figure 11 four binary constructs containing either *Cf*-9 or *Cf*-4 genes under control of the 35S promoter (35S), in combination with either of the *C. fulvum* avirulence genes *Avr9* or *Avr4*. Avirulence genes 25 were also under control of the 35S promoter; transcription termination sequences were provided by the *A. tumefaciens* octopine synthase gene 3' untranslated region (ocs3'). Only restriction enzyme

sites relevant to the construction of each plasmid (see text for details) are shown.

*Positional cloning of the tomato Cf-4 gene.*

*(1) Cf-4 map location.*

5 We have mapped several genes conferring resistance to *C. fulvum* on the classical and RFLP maps of tomato (Dickinson et al., 1993; Jones et al., 1993; Balint-Kurti et al., 1994; Thomas et al., 1995). These studies demonstrated that *Cf-4* and *Cf-9* map to a 10 similar location within a 6 cM interval delimited by two RFLP markers CP46 and TG236 on the short arm of chromosome 1. *Cf-4* and *Cf-9* have been introgressed into cultivated tomato from its wild relatives *L. hirsutum* and *L. pimpinellifolium* respectively (see 15 Jones et al., 1993). Our genetic analysis suggests these genes are tightly linked, or possibly allelic. We have isolated *Cf-9* by transposon tagging using the maize transposon Dissociation (Jones et al., 1994; PCT/GB/02812) and this has enabled us to isolate *Cf-4* 20 by positional cloning.

*(2) Identification of candidate Cf-4 genes.*

DNA from NILs of tomato containing *Cf-9*, *Cf-4* or no known resistance genes to *C. fulvum* were isolated and digested with restriction enzyme *Bgl*II. Southern 25 hybridization analysis with the probe pCf9XS

encompassing the 5' end of *Cf-9* (Jones et al., 1994) revealed extensive RFLPs between the 3 lines (Figure 1). This result was predicted since these resistance genes have been introgressed from different species and 5 since multiple members of this gene family are present on the same introgressed chromosomal segment.

In order to identify a candidtae *Cf-4* gene we intercrossed the *Cf4* and *Cf9* NILs to generate a *Cf-4/Cf-9* transheterozygote. Progeny were subsequently 10 crossed to *Cf0* to generate an *F<sub>1</sub>* test cross population. Approximately 7,500 progeny were inoculated with *C. fulvum* race 5 which is incompatible on plants containing either *Cf-4* or *Cf-9*. We reasoned that if *Cf-4* and *Cf-9* are allelic genes, intragenic 15 recombination could occur in the transheterozygous parent, albeit at a low frequency. In some cases this might remove the resistance specificities of each gene. Progeny containing such recombinant chromosomes would be detected in our analysis as disease-sensitive 20 individuals.

Alternatively, if *Cf-4* and *Cf-9* are not allelic but only closely linked, intergenic recombination could occur, again at low frequency, to generate recombinants either containing both resistance genes or 25 neither. These latter recombinant classes could also be generated as a consequence of chromosomal mis-pairing at meiosis and unequal crossing over. In our analysis only progeny lacking *Cf-4* and *Cf-9* were

identified. Five such disease-sensitive recombinants were detected in 7,500 test cross progeny. These individuals were self-pollinated and progeny were again tested with *C. fulvum* race 5 to confirm they had lost 5 both *Cf-4* and *Cf-9*. Progeny were also identified which were homozygous for the recombinant chromosome. This was performed to simplify subsequent Southern hybridization analysis and was achieved by identifying individuals homozygous for the *Cf9* allele of the RFLP 10 marker CP46 located 2.5 cM distal to *Cf-9* (Balint-Kurti et al., 1994; Thomas et al., 1995).

Southern hybridization analysis of *Bgl*II digested DNA from the 5 disease-sensitive individuals (V408, V512, V514, V516 and V517) using p*Cf9XS* as probe 15 confirmed that as predicted, all lacked the 6.7 kilobasepair (kbp) *Bgl*II band corresponding to the *Cf-9* gene (Jones et al., 1994) and a number of other bands located proximal to the gene. Two other *Bgl*II bands comprising different members of this gene family and 20 located distal to *Cf-9* were present in some recombinants but not others (Figure 2). Three *Bgl*II bands present in *Cf-4* containing lines (6.4 kbp, 3.2 kbp and 1.8 kbp, Figure 1) were also consistently absent from disease-sensitive individuals and were thus 25 candidates for the *Cf-4* gene. This latter result is consistent with analysis of *F*<sub>2</sub> plants from the cross *Cf4* x *L. pennellii*. Specific classes of *F*<sub>2</sub> individuals recombinant in the TG236/CP46 interval were

identified by PCR analysis of leaf material as described previously (Balint-Kurti et al., 1994; Thomas et al., 1995). These results demonstrated the 3 *Bgl*II restriction fragments cosegregate with *Cf-4*. Using 5 Southern hybridization and AFLP analysis we could distinguish 3 classes of recombinants which are depicted in Figure 2. These results are consistent with, but not proof of, a model of chromosomal mis-pairing and unequal crossing over to generate 10 recombinant chromosomes lacking both *Cf-4* and *Cf-9*.

*(3) Isolation of binary vector cosmid clones containing *Cf-9* homologous sequences.*

A genomic DNA library was constructed from a stock that carried both the *Cf-4* gene on chromosome 1, 15 and the *Cf-5* gene on chromosome 6, so that the library could be used for isolating both genes. The library was constructed in a binary cosmid cloning vector pCLD04541, obtained from Dr C. Dean at the John Innes Centre, Norwich (see Bent et al., 1994). The use of 20 such a cloning vector is advantageous since any clones that are isolated can be introduced directly into plants to test for the function of the cloned gene.

High molecular weight DNA was isolated from leaves of 6 week old greenhouse-grown plants by 25 techniques well known to those skilled in the art (Thomas et al., 1994) and partially digested with *Mbo*I restriction enzyme. The partial digestion products

were size fractionated using a sucrose gradient and DNA in the size range 20-25 kbp was ligated to *Bam*HI digested pCLD04541 DNA, using techniques well known to those skilled in the art. After *in vitro* packaging

5 using Stratagene packaging extracts, the cosmids were introduced into a tetracycline sensitive version of *Escherichia coli* strain SURE™ (Stratagene).

Recombinants were selected using the tetracycline resistance gene on pCLD04541.

10 The library was randomly distributed into 144 pools containing about 1500 clones per pool, cells were grown from each pool and from 10 ml of cells, 9 ml were used for bulk plasmid DNA extractions, and 1 ml was used after addition of 0.2 ml of glycerol, to prepare a

15 frozen stock. Plasmid DNA from the pools was isolated by alkaline lysis (Birnboim and Doly, 1979), and DNA samples were subjected to PCR analysis using the primers F7 and F10 which were used to amplify the pCf9XS fragment from a Cf-9 containing tomato line

20 (Jones et al., 1994). Pools 108, 129 and 138 from this library were identified as positive pools for the F7/F10 PCR product. For each pool, approximately 10,000 colonies were plated out and probed by colony hybridisation with radioactively labelled pCf9XS. From

25 each pool, single homologous clones were isolated.

These techniques are well known to those skilled in the art.

The 3 cosmid clones designated 4/5-108, 4/5-129

and 4/5-138 were further characterized by Southern blot hybridisation using pCf9XS as probe and by restriction enzyme mapping. The physical relationships of the 3 cosmids are shown in Figure 3. This analysis showed 5 there were 3 regions showing high homology with pCf9XS located on *Bgl*II restriction fragments of 6.4, 3.2 and 1.8 kbp consistent with results described previously (Figures 1 and 2). These 3 regions were subcloned as *Pst*I fragments from the appropriate cosmid clones 10 (Figure 3) and their DNA sequences determined using techniques well known to those skilled in the art, with oligonucleotide primers previously used to determine the *Cf-9* genomic sequence (Jones et al., 1994) in addition to some new primers specific for each of the 3 15 homologues. The DNA sequences of Homologues I and II were determined entirely from *Pst*I clones derived from cosmids 4/5-108 and 4/5-129 respectively. DNA sequence analysis of a *Pst*I fragment from cosmid 4/5-129 encompassing Homologue III suggested this cosmid does 20 not contain a complete copy of this gene. Physical mapping data suggests this sequence is truncated to a greater extent on cosmid 4/5-138 (Figure 3). This interpretation was confirmed by DNA sequence analysis of a cDNA clone of Homologue III isolated from a 25 *Cf-4/Cf-5* cDNA library. This analysis showed that the copy of Homologue III on cosmid 4/5-129 was truncated by 74 amino acids at its C-terminus. The predicted amino acid sequences of Homologue I (862 amino acids)

Homologue II (806 amino acids) and Homologue III (855 amino acids) all show high levels of homology to Cf-9 (86.5%, 91.5% and 86.5% identical amino acids respectively).

5     *(4) Development of an efficient assay for Cf-4 gene function in plants.*

The function of a putative cloned Cf-4 gene in transgenic plants can be assessed in a number of ways; firstly by inoculation with a race of *C. fulvum* 10 containing the corresponding avirulence gene *Avr4* to test if that race gives an incompatible response on the transgenic plant; secondly by injecting leaves of a transformed plant with intercellular fluid isolated from a compatible interaction containing *AVR4*; thirdly, 15 by delivering *AVR4* in the form of recombinant potato virus X as described previously in studies of the Cf-9/AVR9 interaction (Hammond-Kosack et al., 1995).

The DNA sequence of the *C. fulvum* gene encoding *AVR4* has been reported and the amino acid sequence of 20 the mature processed polypeptide (Joosten et al., 1994). We amplified by PCR the *Avr4* gene from *C. fulvum* race 2,5 using primers to the published sequence and fused a sequence encoding the proposed mature 25 polypeptide to a DNA sequence encoding the N-terminal signal peptide of the tobacco PR1a protein. This would facilitate targeting of *AVR4* to the intercellular space in transgenic plants where it is expressed. This

chimeric gene (*SPAvr4*) was inserted into a cDNA copy of potato virus X, as a *Cla*I/*Sal*I DNA fragment (Figure 4) as described previously (Hammond-Kosack et al., 1995) to generate PVX:*SPAvr4*. Infectious transcripts of the 5 recombinant virus were generated by in vitro transcription. All nucleic acid manipulations were performed using standard techniques well known to those skilled in the art.

Control experiments were designed to test the 10 recombinant virus in 3 week old tomato seedlings. In *Cf-4* containing plants inoculated cotyledons appeared desiccated and eventually abscised at 3 days post-inoculation (d.p.i.), in contrast to *Cf0* controls which only showed signs of slight mechanical damage at 15 the site of virus inoculation. *Cf0* plants developed visible symptoms of virus infection at 7-10 d.p.i. comparable to symptoms observed with the wild type virus i.e. chlorotic mosaic symptoms. At 4-5 d.p.i. in plants containing *Cf-4* necrotic lesions were 20 observed in the younger leaves presumably due to systemic spread of the virus as described previously in similar experiments with PVX containing *Avr9* on *Cf-9* containing plants (Hammond-Kosack et al., 1995). Other features included necrotic sectors on petioles and the 25 stem. The necrotic phenotype was seen to spread systemically and at 14 d.p.i. the majority of *Cf-4* containing seedlings had died. *Cf0* control plants did not die but did show symptoms of chlorosis and

vein-clearing. This procedure therefore, provides a rapid and reliable assay for *Cf-4* function in plants and was applied to transgenic plants to identify individuals potentially carrying the *Cf-4* gene.

5      *(5) Identification of binary vector cosmid clones containing a genomic copy of the Cf-4 gene.*

To test if any of the 3 binary vector cosmid clones (4/5-108, 4/5-129 and 4/5-138) isolated from the *Cf-4/Cf-5* binary vector cosmid library contained *Cf-4*, 10 transgenic tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum* cv Petite Havana) plants were produced (Fillatti et al., 1987; Horsch et al., 1985) using techniques well known to those skilled in the art.

15      Transgenic plants were propagated by cuttings so that *Cf-4* activity could be detected by inoculation with PVX:SPAvr4 on 12 tomato transformants. No transgenic tomato plants (0/3) containing cosmid 4/5-108 exhibited PVX:SPAvr4 dependent leaf necrosis.

20      In contrast, 6 out of 8 transformants containing cosmid 4/5-129 and 3 out of 4 containing cosmid 4/5-138 did exhibit leaf necrosis on inoculated leaves 3-4 d.p.i. (Table 1). This necrosis eventually spread systemically as previously observed in *Cf-4* control 25 plants. Transgenic plants exhibiting necrotic leaf sectors eventually died. Cuttings of a number of transgenic plants obtained in the first round of

transformation experiments were further assayed for Cf-4 function by inoculation with *C. fulvum* race 5 (Table 1).

In these tests a positive correlation was observed between transgenic plants exhibiting PVX:SPAVR4 dependent necrosis and ones resistant to *Cladosporium fulvum* race 5 (Table 1). Pathogen growth was observed on compatible control plants (Cf0) but not on incompatible control plants (Cf2). Self progeny of several transgenic were again tested with *C. fulvum* race 5 to confirm that the trait was heritable. This was shown to be true and in most cases resistance segregated in an approximate ratio of 3:1 consistent with single locus T-DNA transformants (Table 2).  
Transgenic progeny resistant to *C. fulvum* race 5 did not confer resistance to race 4, as predicted since this race does not express the AVR4 peptide (Joosten et al., 1994). This demonstrates that the resistance is a consequence of AVR4 peptide recognition.

Homologue III is completely absent from cosmid 4/5-108 and only truncated copies of this gene are present on the other two cosmids. Therefore Homologue III is an unlikely candidate for Cf-4. It is possible that more than one gene at this locus confers recognition of the AVR4 peptide and pathogen resistance, as occurs in the case of Cf-2 (Dixon et al., 1996). In this latter example however, the two genes are almost identical in contrast to the deduced

amino acid sequences of the 3 *Cf-9* homologous genes described here. A complete copy of homologue I is present in all 3 cosmids (Figure 3). Physical mapping and PCR analysis of cosmid 4/5-108 has shown that 5 Homologue II is truncated in this clone lacking 54 amino acids from its C-terminus as well as the 3' untranslated region and associated transcription termination and polyadenylation sequences.

These data clearly implicate homologue II as the 10 *Cf-4* gene. PVX:SPAvr4 dependent leaf necrosis and resistance to *C. fulvum* race 5 is only apparent in transgenic plants transformed with binary vectors containing complete copies of Homologue II (Table 1). Southern hybridization analysis of a number of 15 resistant primary transformants (4/5-129A, 4/5-129B, 4/5-129D, 4/5-129G, 4/5-129H, 4/5-138A and 4/5-138B) with the pCf9XS probe showed they all contained the 3.2 kbp *Bgl*II DNA fragment characteristic of Homologue II.

That Homologue II specifically recognizes AVR4 is 20 further substantiated by the results of analysis of transgenic *N. tabacum* plant containing these 3 cosmids. When inoculated with PVX:SPAvr4 (Table 3), most transformants containing cosmid 4/5-129 (7/10) and cosmid 4/5-138 (4/5) exhibited necrotic lesions at the 25 site of virus inoculation 3-4 d.p.i. similar in appearance to lesions which appear in response to virus inoculation in some virus resistant varieties. In these individuals the necrosis is not strictly confined

to local lesions which eventually coalesce and at 7-10 d.p.i. leaf necrosis is apparent over the entire region of virus inoculation. In several transformants the reaction to PVX:SPAvr4 is more acute and the necrotic leaf sectors can be observed at 3-4 d.p.i. (Table 3). Neither of these phenotypes were observed in transgenic tobacco containing cosmid 4/5-108 (0/5, see Table 3) or in non-transformed control plants challenged with PVX:SPAvr4.

10 (6) *Analysis of the protein encoded by homologue II.*

The DNA sequence of homologue II (SEQ ID NO. 1, hereafter referred to as Cf-4) is shown in Figure 5. This sequence contains a long uninterrupted open reading frame which upon conceptual translation encodes 15 an 806 amino acid protein as shown in SEQ ID No. 2 (Figure 6). Nucleic acid sequence homology between Cf-4 and Cf-9 in the 3' flanking regions is extremely high and they are identical between the termination codons and the site of polyadenylation of the Cf-9 20 transcript as determined by cDNA analysis (Jones et al., 1994).

Analysis of PCR amplified cDNA clones derived from transcripts of Cf-4 show that it also contains an intron in the 3' untranslated region as in Cf-9 (Figure 25 5).

Comparison of the Cf-4 amino acid sequence with the 863 amino acid sequence of Cf-9 has shown they are

highly homologous (91.5% identity, 95.5% similarity).

As in *Cf-9*, the *Cf-4* amino acid sequence has seven predicted structural domains (Figure 7) as proposed by Jones *et al.*, (1994).

5 Domain A (amino acids 1-23) is consistent with a signal peptide sequence.

Domain B (amino acids 24-81) corresponds to the mature N-terminus of *Cf-4*; this region contains a 10 amino acid deletion relative to *Cf-9* (Figure 8).

10 Domain C (amino acids 82-702) contains 26 imperfect copies of a 24 amino acid LRR sequence. This region contains a 46 amino acid deletion relative to *Cf-9* corresponding to 2 complete LRRs beginning in LRR 10 of *Cf-9* and finishing in LRR 12 (Figure 7). Most of 15 the amino acid variation between *Cf-9* and *Cf-4* is also located in the N-terminal half of the protein (Figure 7). This probably represents the region in each protein which interacts specifically with the cognate avirulence peptides or with other factors which bind 20 the appropriate avirulence peptides.

In the sequences C-terminal to this region the predicted *Cf-4* and *Cf-9* proteins are highly homologous; amino acids 455-806 of *Cf-4* and 512-863 of *Cf-9* (Jones *et al.*, 1994) are identical.

25 Domain D (amino acids 703-730) has no conspicuous features.

Domain E (amino acids 731-748) is markedly acidic containing 10 negatively charged residues.

Domain F (amino acids 749-785) is very hydrophobic and is consistent with a transmembrane domain.

Domain G (amino acids 786-806) is markedly basic 5 with 8 positively charged residues and only 2 negatively charged residues.

The Cf-4 protein, as with Cf-9, has many of the features predicted for a cell-membrane anchored extracytoplasmic protein.

10 (7) *Tomato transgenics which express AVR4 peptide.*

Previous studies have shown that progeny of Cf-9 containing plants crossed to transgenics expressing AVR9 (Hammond-Kosack et al., 1994) exhibit developmentally regulated plant death. Germinating 15 seedlings are phenotypically indistinguishable from their wild type counterparts until 10 days post germination (dpf) when necrosis is observed on cotyledons. The necrosis is progressive and at 15 dpf the seedlings die.

20 We cloned the SP:AVR4 cassette (Figure 4) into the vector SLJ4K1 (Jones et al., 1992) as a *Cla*I/*Bam*HI fragment to provide plant promoter (CaMV 35S) and transcription termination (*nos*3') control sequences for stable expression. This clone was named pAVR4/4K1. 25 The 35S:Avr4 nos3' cassette was excised as a *Bgl*II/*Hind*III fragment and cloned into the binary transformation vector SLJ7291 (Figure 9) digested with

*Bam*HI and *Hind*III to generate the plasmid pAVR4/7291.

Transgenic plants were generated and screened for AVR4 expression by test crossing to the line Cf4. Test cross progeny were screened for the seedling lethal 5 phenotype. Progeny from crosses with six independent transformants were identified which segregated 1:1 for wild type and necrotic seedlings (AVR4/7291J, AVR4/7291O, AVR4/7291L, AVR4/7291N, AVR4/7291M and AVR4/7291G).

10 In these progeny the developmental pattern of necrosis was essentially similar to that described in plants expressing AVR9 and Cf-9.

In progeny of another cross between AVR4/7291G (female parent) and a primary transformant expressing 15 Cf-4 (4/5-129H) seedling lethality was observed in 25% of progeny (35 wild type:10 necrotic) as predicted for an intercross between single locus hemizygous plants. These seedlings were germinated in nutrient agar in a growth room and the pattern of necrosis was different 20 from the control crosses described above. Seedlings which eventually exhibited necrosis were stunted relative to their wild type siblings and necrosis was apparent at an earlier developmental stage.

Furthermore necrotic sectors were also observed in 25 roots, a phenotype not observed in plants expressing Cf-9 and AVR9. In experiments where progeny of 4/5-129H were inoculated with PVX:SPAvr4, necrosis was apparent earlier than in Cf4 control plants. This

phenomenon may reflect elevated *Cf-4* expression in transformant 4/5-129H as a consequence of the chromosomal location of T-DNA insertion.

This result suggests that if sufficient levels of 5 *Cf-4* can be expressed in non-photosynthetic tissue, such as roots, necrosis can be induced in the presence of AVR4. This result therefore has implications for the use of two-component systems to engineer disease resistance, e.g. WO95/31564, in different tissues in a 10 wide range of plants, such as crop plants, and in particular for engineering resistance to root pathogens such as root colonising fungi or nematodes.

**(8) Tomato transgenics containing only Homologue II.**

To further test the conclusion that Homologue II 15 is responsible for AVR4 recognition and resistance to *C. fulvum* race 5, tomato transgenics were generated with Homologue II under control of its own promoter or a promoter capable of directing high level gene expression in plants, i.e. the cauliflower mosaic virus 20 35S promoter (35S, see Jones et al., 1992). These constructions involved DNA manipulations and modifications well known to those skilled in the art.

The 6.0 kbp *Pst*I fragment from cosmid 4/5-129 (129P1, see Figure 3) was cloned into a modified pUC119 25 vector lacking *Eco*RI and *Hind*III restriction sites from the polylinker sequence to generate clone p129P6A. Oligonucleotide mutagenesis was first performed on this

clone to remove internal *Xba*I and *Eco*RI restriction sites commencing at nucleotides 308 and 312 respectively (Figure 5) to generate clone p129P6A-3. These alterations did not alter the predicted amino acid composition of the Cf-4 protein.

To express Homologue II under its own promoter the 6.0 kbp *Xba*I/*Bam*HI cassette from p129P6A-3 was excised and cloned into *Xba*I/*Bam*HI digested SLJ7291 (Figure 9) to generate clone pCf4XB/7291. Further manipulations to p129P6A-3 were performed by oligonucleotide mutagenesis for construction of a vector which might give high level Cf-4 expression in plants. Again, none of the modifications alter the predicted amino acid composition of the Cf-4 protein and all modifications were verified by DNA sequence analysis of the completed construct.

These alterations included the following additional modifications: (i) introduction of a *Cla*I restriction site (ATCGAT) commencing at nucleotide 197 (Figure 5) to facilitate fusion to the 35S promoter in construct SLJ4K1 (Jones et al., 1992); (ii) elimination of an internal *Hind*III restriction site commencing at nucleotide 1766; (iii) elimination of an internal *Bgl*II site commencing at nucleotide 2096; (iv) elimination of a *Cla*I restriction site in the 3' untranslated region commencing at nucleotide 2685.

The modified Homologue II coding sequence and 3' untranslated region was excised as a *Cla*I/*Bam*HI

fragment and cloned into SLJ4K1 (Jones et al., 1992) to produce the plasmid p4K1/Cf4. The 35S:Cf-4 cassette was excised as a *Bgl*III/*Hind*III fragment and cloned into *Bam*HI/*Hind*III cut SLJ7291 to generate plasmid

5 p35SCf4/7291. Tomato transgenics were generated with both binary vector clones and tested by inoculation with PVX:SPAvr4 or *C. fulvum* race 5 as described above.

Several independent transformants containing pCf4XB/7291 exhibited systemic necrosis when inoculated 10 with PVX:SPAvr4 (Table 4). Two out of three transgenics tested were also fully resistant to *C. fulvum* race 5. Eight transgenics containing p35SCf4/7291 exhibited PVX:SPAvr4 dependent necrosis and several tested plants also appeared to give full 15 resistance to *C. fulvum* race 5 (Table 4).

These results demonstrate that Homologue II is necessary and sufficient for AVR4 recognition and to confer full resistance to *C. fulvum* race 5.

**(9) Tobacco transgenics containing only Homologue II.**

20 Previous experiments have shown that the seedling lethal phenotype observed in tomato progeny expressing Cf-9 and AVR9 can also be observed in tobacco. Binary vector plasmids were constructed containing either of two *Pst*I fragments subcloned from cosmid 4/5-129 25 (Figure 3). These fragments were cloned into the plasmid SLJ755A using techniques well known to those skilled in the art to generate plasmids p129P1/755 and

p129P2/755. The binary vector SLJ755A contains the *Streptomyces hygroscopicus* BAR gene under control of the *A. tumefaciens* nos gene promoter (Figure 9). This gene is used as a transformation marker in plants by 5 conferring resistance to the herbicide phosphinotricin (Jones et al., 1992).

These constructs were used successfully in tobacco transformation experiments but not tomato; for tomato transformation alternative vectors were used 10 (see above).

Transgenic tobacco plants were tested for Cf-4 activity by inoculation with PVX:SPAvr4 to monitor the appearance of necrotic lesions or sectors as observed in the binary cosmid vector transformation experiments 15 described above. None of the seven transgenics containing p129P2/755A showed characteristic necrotic lesions or sectors in contrast to ten out of thirteen transgenics containing p129P1/755A (Table 5).

This result is again consistent with the previous 20 observations that Homologue II confers Cf-4 function.

Tobacco transgenics containing pAVR4/7291 (see above) were also generated. Primary transformants containing p129P1/755A expressing Cf-4 were crossed (as female parents) to pAVR4/7291 transgenics to identify 25 AVR4 expressing individuals by monitoring seedling lethality in F1 progeny. Several transgenics expressing AVR4 were identified (AVR4/7291 I, AVR4/7291 K and AVR4/7291 J).

In  $F_1$  progeny of all crosses tested (Table 5) lethality was observed in 25% of seedlings as predicted for progeny of single T-DNA locus hemizygous parents if both transgenics are required for lethality. No variation in the developmental expression of seedling lethality was observed, at least at the macroscopic level, in progeny of the various crosses. However, the phenotype observed in the Cf-4/AVR4 interaction is distinct from the phenotype observed in the Cf-9/AVR9 interaction.

Specifically, tobacco seedling lethality is observed at an earlier stage in seedling development. At seven days post-sowing (on nutrient agar) wild type siblings can be readily distinguished from those expressing Cf-4/AVR4. Cotyledons of seedlings expressing Cf-4 and AVR4 do not expand completely and do not produce appreciable quantities of chlorophyll. In the majority of seedlings the testa remains either attached to one cotyledon or enclosing both cotyledons. This latter feature was not observed in Cf-9/AVR9 expressing tobacco seedlings. At fourteen days post-sowing cotyledons of seedlings expressing Cf-4 and AVR4 appear completely necrotic. These seedlings also fail to develop extensive root systems as reported for the Cf-9/AVR9 interaction.

These results suggest that seedling lethality is expressed at an earlier developmental stage than that described for the Cf-9/AVR9 interaction.

(10) *Transient expression assays to monitor Cf-4 and Cf-9 activity*

Experiments in our laboratory have shown that genes on a binary vector plasmid under control of the 5 CaMV 35S promoter can be transiently expressed in *Nicotiana tabacum* leaf cells when delivered into leaves by infiltrating suspensions of *A. tumefaciens* bacteria. In experiments with 35S:Cf-9 infiltrated leaf panels of stable transgenics expressing AVR9, necrosis was 10 observed in the infiltrated areas. The phenotype was specific to plants expressing AVR9. Other experiments with intron-containing versions of the *uidA* (GUS) reporter gene demonstrated this phenomenon is a consequence of plant nuclear gene expression. The 15 procedure we have used in tobacco was modified from a protocol developed to assay transient gene expression in leaves of *Phaseolus vulgaris*.

Binary vector plasmids were mobilized into *A. tumefaciens* GV3101/pMP90 by tri-parental mating, a 20 technique well known to those skilled in the art. Single colonies grown on L broth medium containing the antibiotics rifampicin (50 µg/ml) and tetracycline (1 µg/ml) were picked and grown in 5 ml L-broth containing antibiotics for 48 h at 28°C in a shaking incubator. 25 One ml of this saturated culture was inoculated into 100 ml L-broth containing antibiotics, 10 mM(2-[N-Morpholino]ethane sulfonic acid (MES) pH 5.6 and 20 µM acetosyringone to induce *Agrobacterium Vir* gene.

Cultures were grown for 16 h at 28°C in a shaking incubator. Bacterial cells were pelleted by low speed centrifugation and resuspended in a buffer containing Murashige and Skoog (MS) salts, 2% w/v sucrose, 500 µM MES pH 5.6 and 10 µM acetosyringone. The OD<sub>600</sub> of each culture was determined and adjusted to an OD<sub>600</sub> of 0.5. Cultures were then incubated without shaking for 3 h at 22°C.

Mature leaves of *N. tabacum* were infiltrated with *Agrobacterium* suspensions using a syringe after several small incisions had been made in the target leaf panel. After injection of several panels infiltrated leaves were covered in a plastic bag to prevent desiccation. After 72 h the bag was removed.

Similar experiments were performed using *Agrobacterium* containing a 35S:Cf-4 binary vector, infiltrated into leaves of *N. tabacum* constitutively expressing AVR4 (see section above). A binary vector containing 35S:Cf-4 was constructed as follows.

The 1.8 kbp *Clal/BamHI* fragment encoding the *uidA* (GUS) gene from *E. coli* was replaced in binary vector SLJ10080 with the 2.9 kbp *Clal/BamHI* fragment encoding Cf-4 derived from plasmid p4K1/CF4 (see Section 8). Similar modifications involving oligonucleotide mutagenesis of Cf-9 DNA were performed as described above to generate a similar construct. The resulting binary vector plasmids pCf4/10080 and pCf9/10080 were mobilized into *A. tumefaciens*.

In transient expression assays *Agrobacterium* suspensions containing pCf4/10080 and pCf9/10080 or a control containing no binary vector, all induced no visible necrosis on non-transformed tobacco. Only leaf 5 panels infiltrated with pCf9/10080 induced a necrotic response on AVR9 expressing tobacco 5-6 days post-infiltration. In the transgenic plant AVR4/7291 K (see Section 9) only leaf panels injected with pCf4/10080 induced a visible necrosis 5-6 days post-infiltration.

10 This assay therefore, provides a quick and reliable procedure to test both *Cf-9* and *Cf-4* gene function in other species, and the effect of specific mutations without the need to generate stable transgenic plants expressing the modified *Cf* genes.

15 We also reasoned that in transient assays, vectors carrying both the 35S:*Cf-4* or 35S:*Cf-9* gene construct and the corresponding 35S:*Avr* gene construct, may result in a visible necrosis in infiltrated leaves. If so, the assay may provide a method to determine the 20 range of species in which *Cf-4* and *Cf-9* can function but without the need to generate transgenic plants expressing either the resistance gene or avirulence gene component. This could have important implications in utilization of a *Cf*-gene/avirulence gene two- 25 component system to engineer resistance in other crop species WO95/31564.

Binary vector plasmids containing both the resistance gene and the avirulence gene were

constructed as follows. The 35S:Cf-4 and 35S:Cf-9 cassettes were excised from pCf4/10080 and pCf9/10080 as follows: (i) digestion with *Apa*I and T4 DNA polymerase treatment to blunt end the DNA fragments; 5 (ii) digestion with *Pst*I to release the 35S:Cf-4 and 35S:Cf-9 cassettes; (iii) the vector SLJ456 (Jones et al., 1992) was linearized with *Cla*I and treated with T4 DNA polymerase to blunt end the DNA and subsequently digested with *Pst*I to release the 2.1 kbp neomycin 10 phosphotransferase (NPT) reporter gene fragment; (iv) the 4.3 kbp 35S:Cf-4 and 4.5 kbp 35S:Cf-9 *Apa*I(T4)/*Pst*I fragments were cloned into *Cla*I(T4)/*Pst*I treated SLJ456 to generate plasmids p4/456 and p9/456. All these techniques are well known to those skilled in the art.

15 Avirulence gene constructs were made as follows.

The clone pAVR4/4K1 (see Section 9) was digested with *Eco*RI and *Bam*HI to excise the 35S:AVR4 cassette. The purified fragment was ligated to the approximately 3.9 kbp *Eco*RI/*Bam*HI fragment from SLJ6B1 (Jones et al., 20 1992) to generate the plasmid pAVR4/6B1 containing 35S, AVR4 and the transcription terminator of the *A. tumefaciens* octopine synthase gene (ocs3'). A 35S:Avr0:ocs3' construct was made in a similar way from the clone SLJ6071 (Hammond-Kosack et al., 1994) to 25 generate plasmid pAVR9/6B1. The plasmids pAVR4/6B1 and pAVR9/6B1 were linearized with *Hind*III and treated with T4 DNA polymerase to blunt end the DNA fragment. The 35S:AVR4:ocs3' and 35S:AVR9:ocs' cassettes were

released by treatment with *Bgl*III. The purified cassettes were each ligated to *Bam*HI/*Hpa*I treated p4/456 and p9/456 to generate four different plasmids in which the function of each resistance gene (*Cf*-4 or 5 *Cf*-9) could be assayed in combination with either AVR4 or AVR9.

Four leaf panels on single leaves of *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* containing each of the four plasmids shown in Figure 10 11. Leaf necrosis was observed five days post-infiltration in panels which received the vector p4/456/Avr4. No necrosis was observed in panels injected with p4/456/Avr9 showing the latter result is a consequence of co-expression of *Cf*-4 and AVR4 in the 15 same cells.

After seven days the necrotic leaves were detached and stored in a sealed polythene bag at room temperature. Necrosis was eventually observed in the leaf panel injected with p9/456/Avr9 but not 20 p9/456/Avr4 twelve days post-infiltration.

These experiments demonstrate that *Cf*- gene necrosis inducing activity can be detected in a transient expression assay in other species when the cognate avirulence determinant is co-expressed. 25 Necrosis induced by the *Cf*-4/AVR4 interaction appeared significantly earlier than that induced by the *Cf*-9/AVR9 interaction. Since all promoter, translation and termination control sequences are the same in each

construct, the latter phenomenon might reflect differences in the relative affinity of Cf-4 and Cf-9 for their corresponding avirulence gene products. This theory would be consistent with the earlier appearance 5 of the seedling lethal phenotype in tobacco transgenics expressing Cf-4 and AVR4 compared to those expressing Cf-9 and AVR9.

<u>Transformant</u>	<u>Response to PVX:SPAvr4</u>	<u>Pathogen inoculation.</u>
4/5-108A	-	S
4/5-108B	-	S
4/5-108C	-	S
4/5-129A	+	R
4/5-129B	+	R
4/5-129C	-	S
4/5-129D	+	R
4/5-129E	+	R
4/5-129F	-	S
4/5-129G	+	R
4/5-129H	+	R
4/5-138A	+	R
4/5-138B	+	R
4/5-138C	-	S
4/5-138D	+	R

**Table 1.** Phenotypes observed in transgenic tomato plants containing cosmids 4/5-108, 4/5-129 or 4/5-138. Plants exhibiting necrotic lesions on inoculated leaves in response to PVX:SPAvr4, which eventually spread systemically are denoted by a (+) sign; plants showing no visible symptoms are denoted by a (-) sign. Plants were also assayed for Cf-4 function by inoculation with *C. fulvum* race 5; R = resistant, S = sensitive.

<u>Tomato genotype</u>	<u><i>C. fulvum</i> (race 5)</u>		<u><i>C. fulvum</i> (race 4)</u>	
	<u>S</u>	<u>R</u>	<u>S</u>	<u>R</u>
<b>Cf4</b>	0	20	15	0
<b>Cf0</b>	20	0	15	0
<b>Cf5</b>	19	0	0	20
<b>4/5-108B</b>	42	0	18	0
<b>4/5-129B</b>	11	23	15	0
<b>4/5-129C</b>	39	0	18	0
<b>4/5-129D</b>	13	29	19	0
<b>4/5-129H</b>	15	20	18	0
<b>4/5-138B</b>	10	28	20	0

Table 2; pathogen inoculation tests on Cf control lines (Cf0, Cf4 and Cf5) and progeny of several primary transformants. Plants were inoculated either with *C. fulvum* race 5 or race 4. Progeny of resistant primary transformants which were previously shown to be resistant to *C. fulvum* race 5 but susceptible to infection by race 4. In most cases progeny segregated at an approximate ratio of 3:1 resistant:susceptible.

<u>Transformant</u>	<u>Symptoms after PVX:SPAvr4 inoculation.</u>
4/5-108A	NVS
4/5-108B	NVS
4/5-108C	NVS
4/5-108D	NVS
4/5-108E	NVS
4/5-129A	Local necrotic lesions
4/5-129B	Local necrotic lesions
4/5-129C	NVS
4/5-129D	NVS
4/5-129E	NVS
4/5-129F	Necrotic leaf sector
4/5-129G	Necrotic leaf sector
4/5-129H	Necrotic leaf sector
4/5-129I	Necrotic leaf sector
4/5-129J	Necrotic leaf sector
4/5-138A	Local necrotic lesions
4/5-138C	Local necrotic lesions + leaf necrosis
4/5-138E	NVS
4/5-138H	Local necrotic lesions
4/5-138J	Local necrotic lesions + leaf necrosis

Table 3. Description of phenotypes observed in transgenic tobacco containing cosmids 4/5-108, 4/5-129 or 4/5-138 at 3-4 days post inoculation with PVX:SPAvr4 (for description of phenotypes see text). NVS = no visible symptoms.

<u>Transformant</u>	<u>PVX: SPAVR4</u>	<u>C. fulvum race 5</u>
Cf4XB/7291A	+	R
Cf4XB/7291B	-	S
Cf4XB/7291C	+	ND
Cf4XB/7291D	+	R
Cf4XB/7291F	+	ND
Cf4XB/7291H	-	ND
35SCf4/7291B	+	R
35SCf4/7291C	(ND)	R
35SCf4/7291D	+	R
35SCf4/7291E	+	R
35SCf4/7291F	+	R
35SCf4/7291G	+	R
35SCf4/7291H	+	ND
35SCf4/7291I	ND	R
35SCf4/7291J	+	R
35SCf4/7291K	+	R
35SCf4/7291P	-	S
35SCf4/7291Q	ND	R

Table 4. Phenotypes observed in transgenic tomato plants containing vectors pCFXB/7291 and p35SCf4/7291. Plants exhibiting necrotic lesions on inoculated leaves in response to PVX:SPAvr4, which eventually spread systemically are denoted by a (+) sign; plants showing no visible symptoms are denoted by a (-) sign. Plants were also assayed for Cf-4 function by inoculation with *C. fulvum* race 5; R = resistant, S = sensitive ND = not determined.

<u>Transgenic</u>	<u>PVX:SPAVR4</u>	<u>Seedling lethality</u>
129P1/755 A	+	AVR4/7291 J
129P1/755 B	+	ND
129P1/755 C	-	ND
129P1/755 D	+	AVR4/7291 K
129P1/755 E	+	AVR4/7291 J and K
129P1/755 F	+	AVR4/7291 J and K
129P1/755 G	+	ND
129P1/755 H	+	ND
129P1/755 I	+	AVR4/7291 I
129P1/755 J	+	ND
129P1/755 L	-	ND
129P1/755 N	ND	AVR4/7291 K, and I
129P1/755 O	+	ND
129P2/755 A	-	ND
129P2/755 B	-	ND
129P2/755 D	-	ND
129P2/755 E	-	ND
129P2/755 G	-	ND
129P2/755 J	-	ND
129P2/755 K	-	ND

**Table 5.** Expression of Homologue II from its own promoter in transgenic tobacco. Transgenics were assayed for *Cf-4* activity by inoculation with PVX:SPAVR4. Plants exhibiting necrotic lesions or sectors are indicated by a (+) and those exhibiting no response by (-). Only transgenics containing Homologue II (129P1/755) conferred *Cf-4* function. Several *Cf-4* expressing plants were subsequently crossed to different transgenics (AVR4/7291 I, J or K) expressing AVR4 as indicated in the Table. Seedlings of all these crosses segregated 3:1 for either a wild type or necrotic phenotype.

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CLAIMS

1. A nucleic acid isolate encoding a pathogen resistance gene whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide comprising the sequence of amino acids shown in SEQ ID NO. 5.
- 2.
  
2. Nucleic acid according to claim 1 wherein said activation is upon contact of the plant with a pathogen or 10 corresponding elicitor molecule.
  
3. Nucleic acid according to claim 1 wherein the sequence of nucleotides comprises an encoding sequence shown in SEQ ID NO. 1.
  
4. Nucleic acid according to claim 1 wherein the 15 sequence of nucleotides comprises an allele, derivative or mutant, by way of addition, insertion, deletion or substitution of one or more nucleotides, of an encoding sequence shown in SEQ ID NO. 1.
  
5. Nucleic acid encoding a pathogen resistance gene 20 whose expression in a plant can cause activation of a defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide, the polypeptide

comprising an amino acid sequence which comprises an allele, derivative or mutant, by way of addition, insertion, deletion or substitution of one or more amino acids, of the amino acid sequence shown in SEQ ID NO. 2;

5 with the proviso that the encoded polypeptide has at least about 95% amino acid sequence identity with the amino acid sequence shown in SEQ ID NO. 2.

6. Nucleic acid encoding a pathogen resistance gene whose expression in a plant can cause activation of a 10 defence response in the plant, comprising a sequence of nucleotides encoding a polypeptide, the polypeptide comprising an amino acid sequence which comprises an allele, derivative or mutant, by way of addition, insertion, deletion or substitution of one or more amino acids, of the amino acid sequence shown in SEQ ID NO. 2;

15 with the proviso that expression of the nucleic acid can cause said activation of a defence response upon contact of the plant with Avr4 elicitor molecule.

7. Nucleic acid according to claim 5 or claim 6 wherein 20 said activation is upon contact of the plant with a pathogen or corresponding elicitor molecule.

8. Nucleic acid which is a vector comprising nucleic acid according to any one of claims 1 to 7.

9. Nucleic acid according to claim 8 further comprising regulatory sequences for expression of said polypeptide.

10. Use of nucleic acid according to any one of the precedings claims in production of a transgenic plant.

5 11. A host cell comprising nucleic acid according to any one of claims 1 to 9.

12. A host cell according to claim 11 which is microbial.

10 13. A host cell according to claim 11 which is a plant cell.

14. A plant or any part thereof comprising a cell according to claim 13.

15. Seed, selfed or hybrid progeny or a descendant or derivative or extract of a plant according to claim 14, or 15 any part thereof.

16. A method which comprises introduction of nucleic acid according to any one of claims 1 to 9 into a host cell.

17. A method according to claim 16 wherein the host cell is a plant or microbial cell.

18. A method of conferring pathogen resistance on a plant, comprising expression from nucleic acid according to any one of claims 1 to 9, within cells of the plant, following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

19. A method according to claim 18 wherein the nucleic acid encodes an amino acid sequence shown in SEQ ID NO. 2.

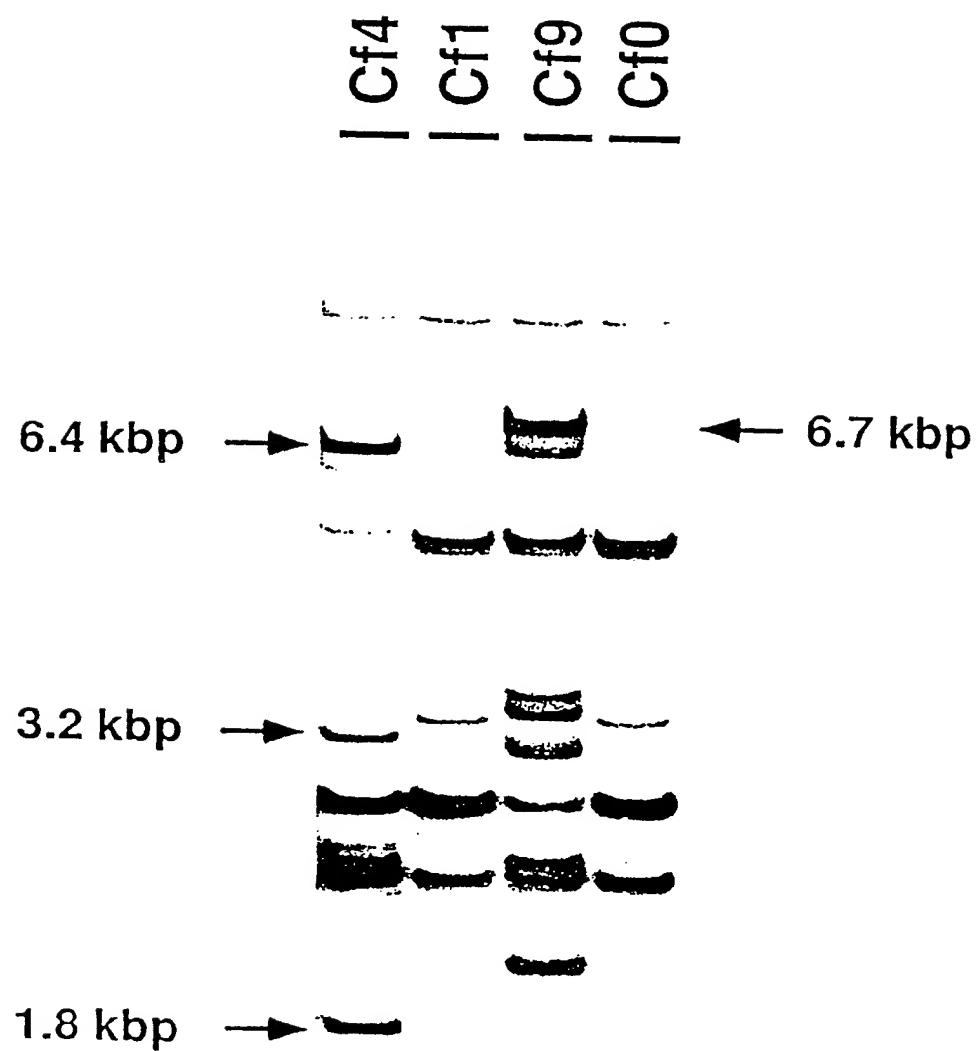


Figure 1

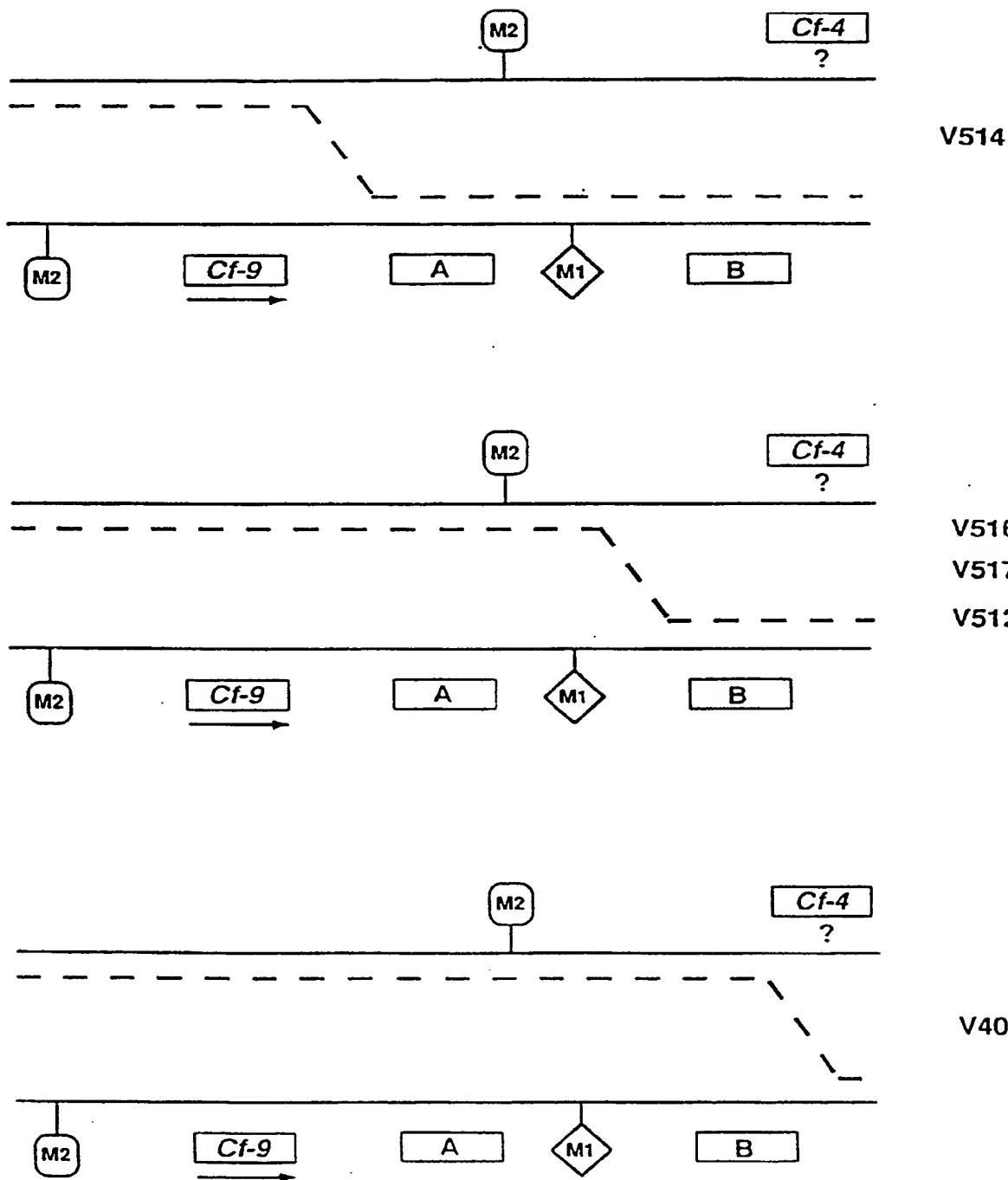
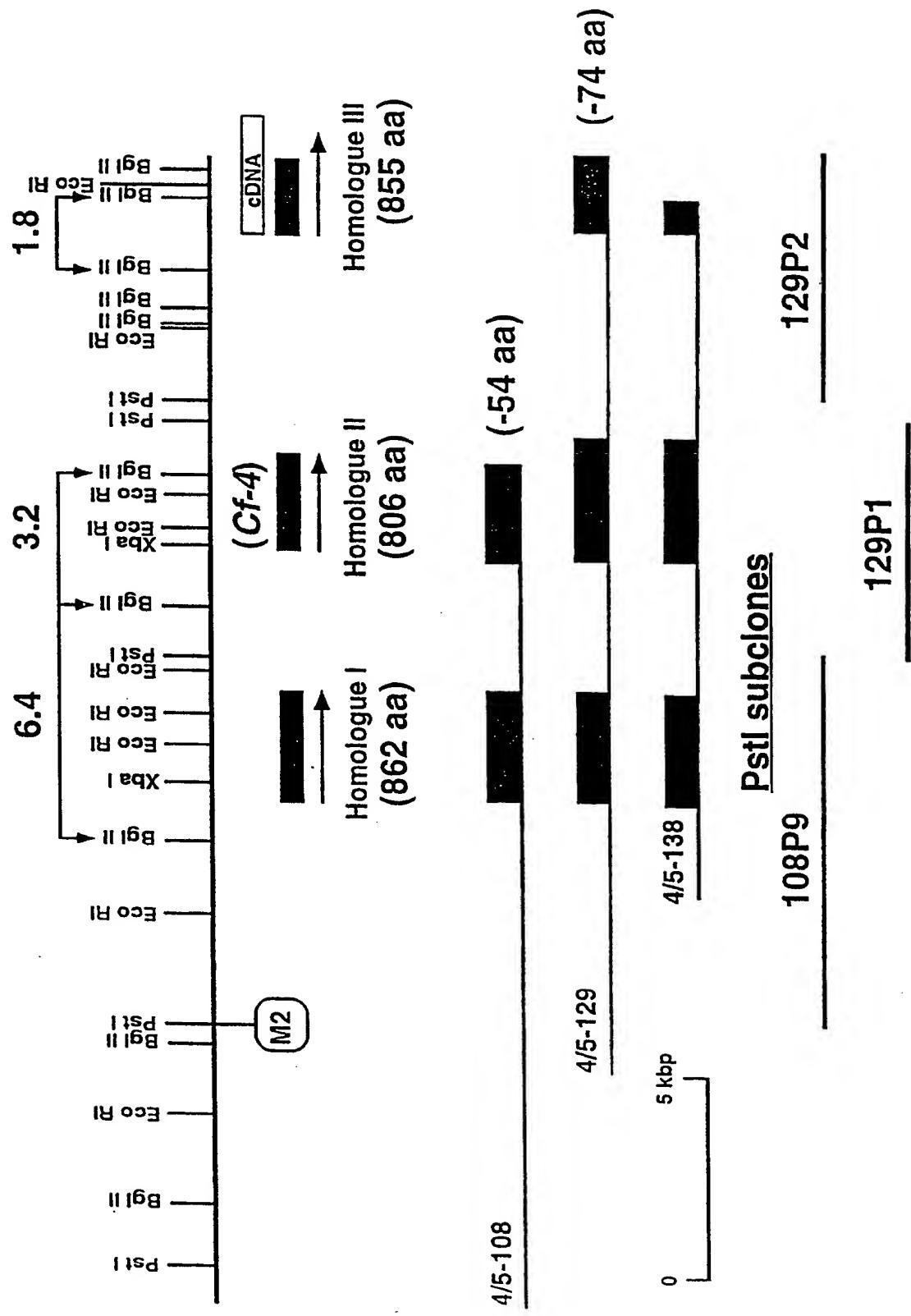


Figure 2



**Figure 3**

1 ATCGATGGGATTGTTCTCTTTACAATTGCCCTCATTCTTCTTGTCTACACTTCT  
 1 TAGCTACCCCTAAACAAGAGAAAAGTGTAAACGGAAGTAAAGAAGAACAGAGATGTGAAGA 60  
 M G F V L F S Q L P S F L L V S T L L  
 61 CTTATTCCCTAGTAATATCCCACCTTGCCGTGCCAAAGCCCCAAAACCTCAACCATAACAA  
 61 GAATAAGGATCATTATAGGGTGAGAACGGCACGGTTTCGGGGTTTGAGTTGGTATGTT 120  
 L F L V I S H S C R A K A P K T Q P Y N  
 121 CCCATGCAAGCCCCAAGAAGTCATGACACCAAGTGTATGGGTCCCAAGGATTGTCTCTA  
 121 GGGTACGTTCGGGGTTCTCAGTAGCTGTGGTTACACATACCCAGGGTCTAACAGAGAT 180  
 P C K P Q E V I D T K C M G P K D C L Y  
 181 CCCGAACCCCGACAGTTGTACAACCTACATACAGTGTGTACCGCTCGACGAAGTTGGCAA  
 181 GGGCTTGGGGCTGTCAACATGTTGGATGTATGTCACACATGGCGAGCTGCTTCAACCGTT 240  
 P N P D S C T T Y I Q C V P L D E V G N  
 241 TGCAGACGCTGTGGTTAAGCCATGTCAAAGGACTGCAGTGGAACGATAACGTTGGCAA  
 241 ACGCTTCGGACACCAATTGGTACAGGTTTCCTGACGTACCTTGTATTGCAACCGTT 300  
 A K P V V K P C P K G L Q W N D N V G K  
 301 GAAGTGGTGCAGTATCCAAACCTGAGTACGTGTCCGGTAAAGACGCCGCAACCGAAGCC  
 301 CTTCACACAGCTGATAGGTTGGACTCATGCACAGGCCATTCTGGCGTTGGCTTCGG 360  
 K W C D Y P N L S T C P V K T P Q P K P  
 361 GAAGAAGGGAGGTGTCGGAGGGAAAGAAGGCGTCGGTTGGACATCCTGGCTATTGAGTCGG  
 361 CTTCTCCCTCCACAGCCTCCCTTCTCCGCAGCCAACCTGTAGGACCGATAACTCAGCC 420  
 K K G G V G G K K A S V G H P G Y  
 421 ACAAGAAAGGGGATGGCTGTAACAGTTCTGGTACCAAGAGCTATCGTGTAGGGATCCGT  
 421 TGTTCTTCCCCTACCGACATTGTCAAGACCATGGTCTCGATAGCACGATCCCCTAGGCA 480  
  
 481 CGAC  
 481 GCTG

Figure 4

## FIGURE 5

1 GACCAAACTG GACTCCTGCT CCGCTTCCA TCAGCAGGTC AATTCTCGTG  
51 GAAAATTAGC TCGAGGTGGC GCACTATGTG AGGTAGCTAG TACTAAATGT  
101 TTATTTGCGT AATTTGTGCT ATATATACTT CATCTAAATT ATTGAATAGA  
151 CACACAAAGC AAACATCTCT TAATTAGTT TGATCATTG TAGTGCAGAA  
201 ATGGGTTGTG TAAAACCTGT GTTTTCATG CTATATGTCT TTCTCTTCA  
251 ACTTGTTC CCGTCATCCT TACCTCATTG GTGCCCGAA GATCAAGCTC  
301 TTGCTCTTCT AGAATTCAAG AACATGTTA CCGTTAATCC TAATGTTCT  
351 GATTATTGTT ACGACAGAAAG AACTCTTCT TGGAACAAAAA GCACAAAGTTG  
401 CTGCTCATGG GATGGCGTTC ATTGTGACGA AACGACAGGA CAAGTGTGATTG  
451 AGCTTGACCT CCGTTGCATC CAACTTCAAG GCAAGTTCA TTCCAATAGT  
501 AGCCTCTTTC AACTCTCCAA TCTCAAAAGG CTTGATTGTT CTTATAATGA  
551 TTTCACTGGA TCGCCCATT CACCTAAATT TGGTGAGTTT TCAGATTTGA  
601 CGCATCTCGA TTTGTCGCAT TCAAGTTTA GGGGTGTAAT CCCTCTGAA  
651 ATCTCTCATC TTTCTAAACT ATACGTTCTT CGTATTAGTC TAAATGAGCT  
701 TACTTTGGT CCTCACAATT TTGAATTGCT TCTTAAGAAC TTGACCCAAT  
751 TAAAAGTGCT CGACCTTGAA TCTATCAACA TCTCTTCCAC TATTCTTTG  
801 AATTCTCTT CTCATTTAAC AAATCTATGG CTTCCATACA CAGAGTTACG  
851 TGGGATATTG CCCGAAAGAG TTTTCCACCT TTCCGACTTA GAATTCTCG  
901 ATTTATCAAG CAATCCCCAG CTCACGGTTA GGTTTCCCAC AACCAAATGG  
951 AATAGCAGTG CATCACTCAT GAAGTTATAT CTCTATAATG TGAATATTGA  
1001 TGATAGGATA CCTGAATCAT TTAGCCATCT AACTTCACCTT CATAAGTTGT  
1051 ACATGAGTCG TTCTAATCTG TCAGGGCCTA TTCTAAACC TCTATGGAAT  
1101 CTCACCAACA TAGTGTGTTT GGACCTTAAT AATAACCATC TTGAAGGACC  
1151 AATTCCATCC AACGTAAGCG GACTACGTA CCTACAAATA CTTGGTTGT  
1201 CATCAAACAA CTAAATGGG AGTATACCAT CCTGGATATT CTCCCTTCCA  
1251 TCACTGATAG GGTTAGACTT GAGCAATAAC ACTTTCAGTG GAAAAATTCA  
1301 AGAGTTCAAG TCCAAAACAT TAAGTACCGT TACTCTAAA CAAAATAAGC  
1351 TAAAAGGTCC TATTCCGAAT TCACTCCTAA ACCAGAAGAA CCTACAATTG  
1401 CTTCTCTT CACACAATAA TATCAGTGGA CATATTTCTT CAGCTATCTG  
1451 CAATCTGAAA ACATTGATAT TGTTAGACTT GGGAAAGTAAT AATTGGAGG  
1501 GAACAATCCC GCAATGCGTG GTTGAGAGGA ACGAATACCT TTGCGATTG  
1551 GATTGAGCA ACAACAGACT TAGTGGGACA ATCAATACAA CTTTTAGTGT  
1601 TGGAAACATT TTAAGGGTCA TTAGCTTGCA CGGGAATAAG CTAACGGGGA

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1651 AAGTCCCACG ATCTATGATC AATTGCAAGT ATTTGACACT ACTTGATCTA  
1701 GGTAACAATA TGTTGAATGA CACATTCCA AACTGGTTGG GATAACCTATT  
1751 TCAATTGAAG ATTTTAAGCT TGAGATCAAA TAAGTTGCAT GGTCCCATCA  
1801 AATCTTCAGG GAATACAAAC TTGTTTATGG GTCTTCAAAT TCTTGATCTA  
1851 TCATCTAATG GATTTAGTGG GAATTACCC GAAAGAATT TGGGGAATT  
1901 GCAAACCATG AAGGAAATTG ATGAGAGTAC AGGATTCCA GAGTATATT  
1951 CTGATCCATA TGATATTAT TACAATTATT TGACGACAAT TTCTACAAAG  
2001 GGACAAGATT ATGATTCTGT TCGAATTTCG GATTCTAACCA TGATTATCAA  
2051 TCTCTCAAAG AACAGATTG AAGGTCATAT TCCAAGCATT ATTGGAGATC  
2101 TTGTTGGACT TCGTACGTG AACTTGTCTC ACAATGTCTT GGAAGGTCA  
2151 ATACCGGCAT CATTCAAAGA TTTATCAGTA CTCGAATCAT TGGATCTCTC  
2201 ATCTAATAAA ATCAGCGGAG AAATTCCGCA GCAGCTTGCA TCCCTCACAT  
2251 TCCTTGAAAGT CTTAAATCTC TCTCACAATC ATCTTGTGG ATGCATCCCC  
2301 AAAGGAAAAC AATTGATTC GTTCGGGAAC ACTTCGTACC AAGGGAATGA  
2351 TGGGTTACGC GGATTCCAC TCTCAAAACT TTGTGGTGGT GAAGATCAAG  
2401 TGACAACCTCC AGCTGAGCTA GATCAAGAAG AGGAGGAAGA AGATTCAACCA  
2451 ATGATCAGTT GGCAGGGGGT TCTCGTGGGT TACGGTTGTG GACTTGTAT  
2501 TGGACTGTCC GTAATATACA TAATGTGGTC AACTCAATAT CCAGCATGGT  
2551 TTTCGAGGAT GGATTAAAG TTGGAACACA TAATTACTAC GAAAATGAAA  
2601 AAGCACAAGA AAAGATATTA GTGAGTAGCT ATACCTCCAG GTATTCCACT  
2651 TGATCATTAT CTTTCAGAAG ATTATTTTT GTATATCGAT GAAATTATCG  
2701 ACCTCTTCA TCCTCAAAGC TCTTAACCTT CACTCTTCAT TTTGAAAAT  
2751 TTCAGGATTC AAAGATTCC GAGTTCCCAG TTGCTTGGGA TGCAGATAAA  
2801 AGCCTTTTA TCTTCATAG TTTCTTATCC TATGAATAAA GATTTTATTT  
2851 TCATTTGTCT ATGGCACGTA GATATGTCC GTCACTAAAA ACATTGTATT  
2901 TCTCTCAACT CTTTCGTAC ATGATATCAA AGAACACTTG ACTTCAAATTA  
2951 AGTTACTGTA GTCTGCTATT TTAATTCTT CCATTGAAAC ACAACTGACG  
3001 TATCTTGAGA AAGAGACTAT GATCTCAGAA ATGGGAATCT CCCAATCCAA  
3051 AACTCGAAA ATCTAGTATC AAACACACCC GACCCTGCAG

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## FIGURE 6

1 MGCVKLVFFM LYVFLFQLVS SSSLPHLCPE DQALALLEFK NMFTVNPNAS  
51 DCYDRRTLS WNKSTSCCSW DGVHCDETTG QVIELDLRCI QLQGKFHSNS  
101 SLFQLSNLKR LDLSYNDFTG SPISPKFGEF SDLTHLDLSH SSFRGVIPSE  
151 ISHLSKLYVL RISLNELTFG PHNFELLKN LTQLKVLDE SINISSTIPL  
201 NFSSHLTNLW LPYTELRGIL PERVFHLSQL EFLDLSSNPQ LTVRFPTTKW  
251 NSSASLMKLY LYNVNIDDR1 PESFSHLTS1 HKLYMSRSNL SGPIPKPLWN  
301 LTNIVFLDLN NNHLEGPIPS NVSGLRNQI LWLSSNNLNG SIPSWIFSLP  
351 SLIGLDLSNN TFSGKIQEFK SKTLSTVTLK QNKLKGPIPN SLLNQKNLQF  
401 LLLSHNNISG HISSAICNLK TLILLLGSN NLEGTIPQCV VERNEYLSHL  
451 DLSNNRLSGT INTTFSVGN1 LRVISLHGNK LTGKVPRSM1 NCKYLTLLDL  
501 GNNMLNDTFF NWLGYLQQLK ILSLRSNKLH GPIKSSGNTN LFMGLQILDL  
551 SSNGFSGNLP ERILGNLQTM KEIDESTGFP EYISDPYDIY YNYLTTISTK  
601 GQDYDSVRIL DSNMIINLSK NRFEGHIPSI IGDLVGLRTL NLSHNVLEGH  
651 IPASFQNL1V LESLDLSSNK ISGEIPQQLA SLTFLEVNL SHNHLVGCIP  
701 KGKQFDSFGN TSYQGNDGLR GFPLSKLCGG EDQVTTPAEL DQEEEEEDSP  
751 MISWQGVLVG YGCGLVIGLS VIYIMWSTQY PAWFSSRMDLK LEHIIITKMK  
801 KHKKRY

# Cf-4

**A**

MGCVKLVFFMLYVFLFQLVSSSS

**B**

LPHLCPEDQALALLEFKNMFTVNPNASDYCYD...  
.....RRTLSWNKSTSCCSWDGVHCDETTGQ

VIELDLRCIQLQGKFHSNSS  
LFQLSNLKRLLDSYNDFTGSPISP  
FGEFSDLTHLLDLSHSSFRGVIPSE  
ISHLSKLYVLRISLNELTFGPHNFELL  
LKNLTOLKVLDLESINISSTIPL  
NFSSSHLTNWLWLPYTELRGILPER  
VFHLSDEFLDLSSNPQLTVRFPTTK  
WNSSASLMKLYLYNVNIDDRIPES  
FSHLTSLHKLYMSRSNLSGPIPKP  
LWNLTNIVFLDLNNNNHLEG....

**C**

.....PIPSN  
VSGGLRNLQILWLSSNNNGSIPSW  
IFSLPSLIGLDLSNNNTFS GKIQE  
KSKTLSTVTLKQNKLGPIPN  
LLNQKNLQFLLLSHNNISGHISSA  
ICNLKTLILLLDLGSNNLEGTIPQC  
VERNEYLSHLDLSNNRLSGTINTT  
FSVGNILRVISLHGNKLTGKVPRS  
MINCKYLTLLDLGNNMNDTFPNW  
LGYLFQLKILSRNKLHGPIKSGN  
TNLFMGLQILLDLSNGFSGNLPE  
LGNLQTMKEIDESTGPEYISDPY  
DIYNNYLTTI STKGQD YDSVRI  
LDSNMI INLSKNRFEGHIPS  
IGDLVGLRTINLSHNVLEGHIPS  
FQNLSVLESDLSNKISGEIPQQ  
LASLTFLEVLNLSHNHLVGCIPKG

**D**

KQFD~~SFGNT~~SYQGNDGLRGFPLSKLCGG

**E**

EDQVTTPAELDQEEEEED

**F**

SPMISWQGVLVGYGCGLVIGLSVIYIMWSTQYPAWFS

**G**

RMDLKLEHIITTKMKKHKKRY

**Figure 7.**

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**A**

40 KNMFTINPNASD~~Y~~CYDIRTYVDIQSYPRTL~~W~~NKSTSCCS 79 Cf-9  
 |||||:|||||:||||| .|||||:|||||  
 40 KNMFTVNP~~N~~NASD~~Y~~CYD.....RRTL~~W~~NKSTSCCS 69 Cf-4

**B**

LRR 9		LRR 10	
285	FSHLTS <del>L</del> HELYMGR <del>C</del> NLSGP <del>I</del> PKP	LWNLT <del>N</del> IVFL <del>H</del> LD <del>G</del> NHLEGPI <del>S</del> H FTI	334 Cf-9
274	FSHLTS <del>L</del> H <del>K</del> LYMSRSNLSGP <del>I</del> PKP	LWNLT <del>N</del> IVFL <del>D</del> LN <del>N</del> NHLEG .....	315 Cf-4
LRR 9		LRR 10	
LRR 11		LRR 12	
335	FEKLKRL <del>S</del> V <del>N</del> NNFDGGLEF	LSFNTQLERLDLSSNSLTGPIPSN	ISGLQN 384 Cf-9
316	.....	PIPSN	VSGLRN 327 Cf-4
LRR 11		LRR 12	
LRR 13		LRR 14	
385	LECLYLSSNH <del>L</del> NGSIP <del>W</del>	IFSLPSLVELDLSNNTFSGKIQEF	426 Cf-9
328	LQILWLSSNNLNGSIP <del>W</del>	IFSLPSLIGLDLSNNTFSGKIQEF	369 Cf-4
LRR 11		LRR 12	

**Figure 8**

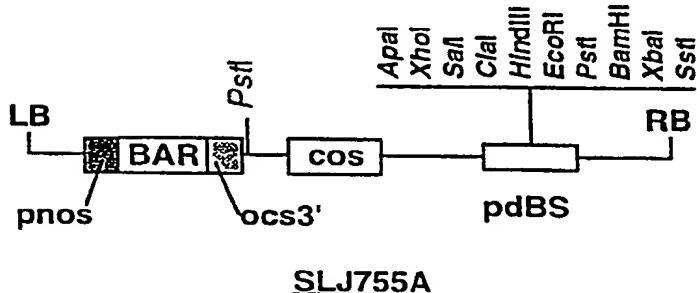
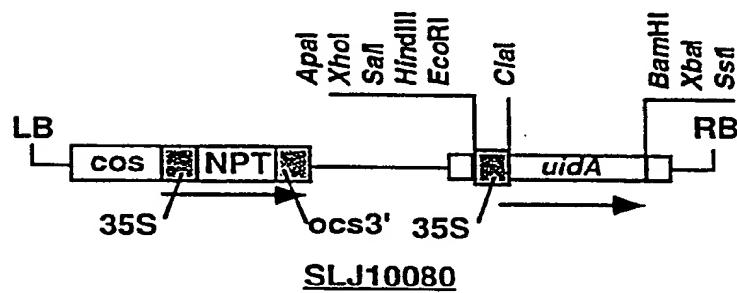
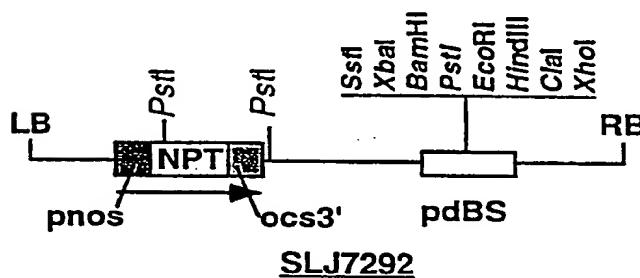
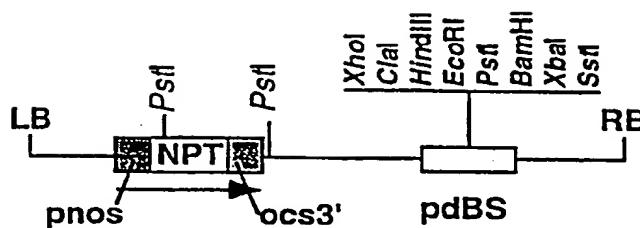


Figure 9

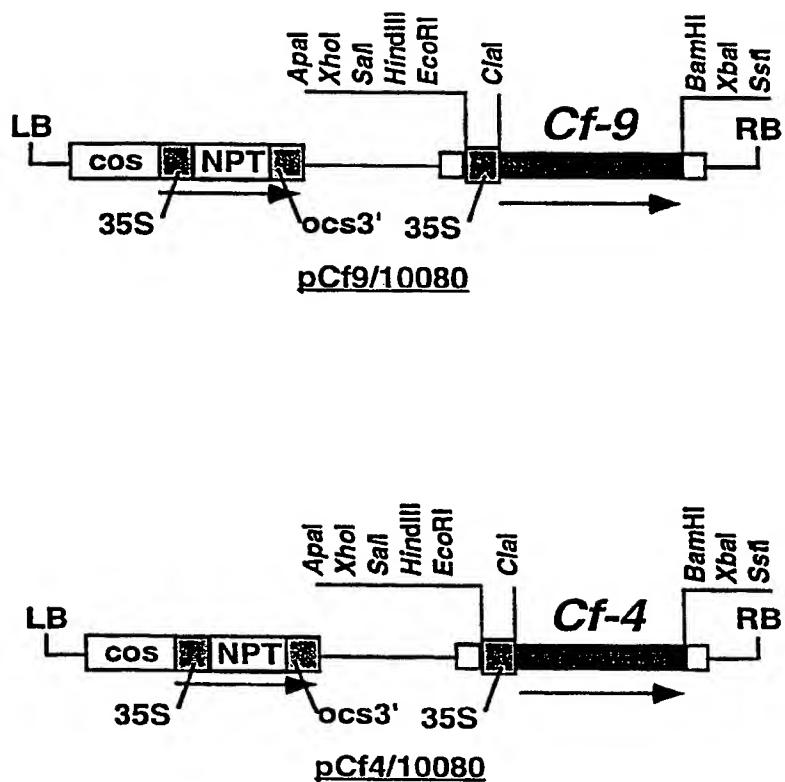


Figure 10

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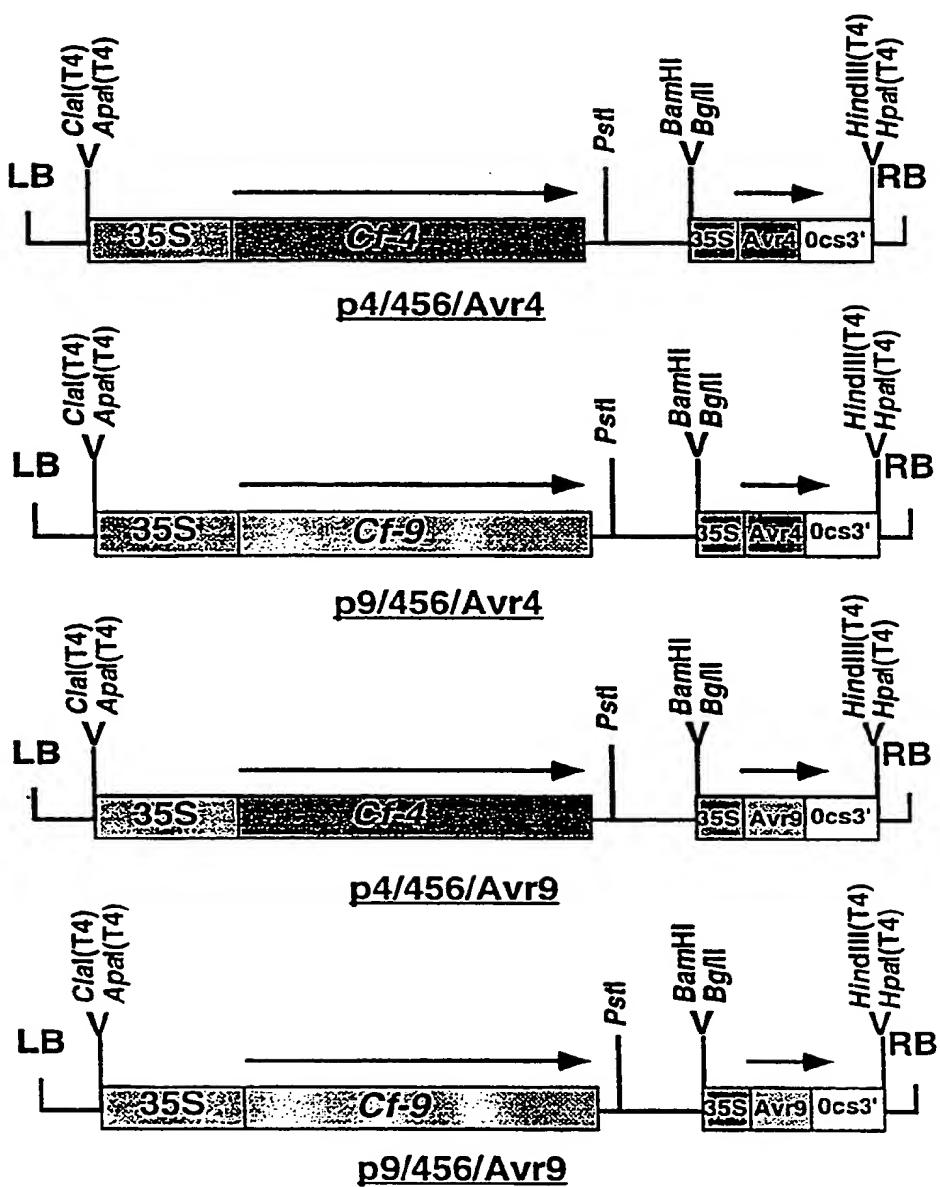


Figure 11

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/01155

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C12N15/29 C12N15/82 A01N65/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12N A01N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO,A,95 31564 (GATSBY CHARITABLE FOUNDATION ; JONES JONATHAN DALLAS GEORGE (GB); H) 23 November 1995 see sequence ID's 11 and 12 see page 44 see page 63 - page 66 ---	1-19
Y	TRENDS IN GENETICS, vol. 11, February 1995, pages 63-68, XP002006911 TANKSLEY, S.D., ET AL.: "Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes." see the whole document ---	1-19 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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3

Date of the actual completion of the international search

27 September 1996

Date of mailing of the international search report

14.10.96

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## INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THEORETICAL AND APPLIED GENETICS 88 (6-7). 1994. 691-700. , XP000574061 BALINT-KURTI P J ET AL: "RFLP linkage analysis of the Cf-4 and Cf-9 genes for resistance to <i>Cladosporium fulvum</i> in tomato." see the whole document ---	1-19
Y	PLANT PHYSIOLOGY, vol. 101, 1993, pages 709-712, XP002006913 CORNELISSEN, B.J.C., ET AL.: "Strategies for control of fungal diseases with transgenic plants" see page 709, left-hand column, last paragraph - page 710, left-hand column, paragraph 1 ---	10,13-19
A	CELL, vol. 80, 10 February 1995, pages 363-366, XP002006912 DANGL, J.L.: "Pièce de Résistance: novel classes of plant disease resistance genes" see page 365, right-hand column, line 13 - line 24 ---	1-19
A	SCIENCE, vol. 266, 4 November 1994, pages 789-793, XP002007124 JONES, D.A., ET AL.: "Isolation of the tomato Cf-9 gene for resistance to <i>Cladosporium fulvum</i> by transposon tagging" see the whole document ---	1-9
A	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT. KEYSTONE SYMPOSIUM HELD MARCH 29-APRIL 4, 1995., vol. 21a, 1995, page 485 XP002007014 DIXON, M.S., ET AL.: "Cloning and characterisation of the Cf-2 disease resistance gene related family members and the corresponding null locus" see abstract J6-203 ---	1-19
A	SCIENCE, vol. 268, 5 May 1995, pages 661-667, XP002007125 STASKAWICZ, B.J., ET AL.: "Molecular genetics of plant disease resistance" see the whole document ---	1-19
3	WO,A,95 05731 (CORNELL RES FOUNDATION INC) 2 March 1995 see claim 31 ---	10,13-19
2		
		-/-

## INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/GB 96/01155

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 118, no. 17, 26 April 1993 Columbus, Ohio, US; abstract no. 161928, JONES, J. D. G. ET AL: "Prospects for establishing a tomato gene tagging system using the maize transposon Activator (Ac)" XP002014516 see abstract &amp; PROC. - R. SOC. EDINBURGH, SECT. B: BIOL. SCI. (1992), 99(3-4), 107-19 , ---</p>	1-19
P,A	<p>WO,A,95 18230 (GATSBY CHARITABLE FOUNDATION ;JONES JONATHAN DALLAS GEORGE (GB); H) 6 July 1995 see the whole document -----</p>	1-19

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Internal Application No

PCT/GB 96/01155

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9531564	23-11-95	AU-A-	2415495	05-12-95
		AU-A-	1321695	17-07-95
WO-A-9505731	02-03-95	NONE		
WO-A-9518230	06-07-95	AU-A-	1321695	17-07-95
		CA-A-	2178488	06-07-95

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